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Identification and Characterization of Mitochondria-rich Cell Sub-types from the Gills of Rainbow Trout

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Identification and Characterization of Mitochondria-rich Cell Sub-types from the Gills of Rainbow Trout" submitted by Guy S. Hawkings in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Cell Biology.

Abstract:

We have developed a technique for isolating at least two populations of morphologically and functionally distinct mitochondria-rich (MR) cells from gills of rainbow trout based on differential binding to peanut lectin agglutinin (PNA). This study investigated ion transport in these MR cell sub-types under different environmental conditions. We found that the relative percentage of these MR cell sub-types is altered during seawater (SW) acclimation. The gills of SW-acclimated trout show independent alterations in Na^+/K^+- and H^+-ATPase activity in these PNA fractions. Additionally, the PNA- (no PNA binding) MR cells have acid induced, phenamil-sensitive Na^+ uptake, as demonstrated by $^{22}Na^+$ fluxes during acid/base stress. Acid-induced Na^+ uptake is blocked by the addition of silver (Na^+ channel competitor/inhibitor). These data support the hypothesis that at least two functionally separate MR cell sub-types exist in the gills of rainbow trout and have lead to a revision of the current freshwater fish gill model.

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Abbreviations

$^{22}Na^{+}$ - radioactive sodium
α - Greek letter alpha
β - Greek letter beta
AE - anion exchanger
Ag^{+} - silver
ATP - adenosine triphosphate
ATPase - adenosine triphosphatase
c.p.m. - counts per minute
CA - carbonic anhydrase
Ca^{2+} - calcium
CCD - cortical collecting duct
CFTR - cystic fibrosis trans-membrane conductance regulator
Cl^{-} - chloride
Cu - copper
DIC - differential interference contrast
ECF - extracellular fluid
ENaC - epithelial sodium channel
FACS - fluorescent-activated cell sorting
FITC - fluorescein isothiocyanate
h - hour
H^{+} - proton
$HCO3^{-}$ - bicarbonate
HRP - horseradish peroxidase
IC - intercalated cells
J_{in}^{Na} - sodium influx
K^{+} - potassium
kBq - kiloBecquerel
LC_{50} - half-maximal lethal concentration
MACS - magnetic cell sorting
MR - mitochondria-rich
Na^{+} - sodium
NBC - sodium/bicarbonate cotransporter
NHE - sodium/proton exchanger
NKCC - Na+/K+/2 Cl- co-transporter
PBS - phosphate buffered saline
P_{CO2} - partial pressure of carbon dioxide
PNA - peanut lectin agglutinin
RBCs - red blood cells
SEM - scanning electron microscopy
TEM - transmission electron microscopy

Chapter 1: General Introduction

Historical Perspectives:

In the early part of the last century, the function of the fish gill was thought to be largely respiratory. However, the ability of specific gill cells to accomplish the movement of ions was first described by Keys and Wilmer (Keys and Willmer, 1932) working with seawater eels. They found that placing silver (Ag) in the water resulted in precipitation of silver chloride (AgCl) complexes on the surface of particular cells of the gill. They proposed that certain gill cells, therein named "chloride cells", actively secrete chloride (Cl^-) in marine teleosts, resulting in AgCl precipitates forming on the gill surface in their assay. Keys and Wilmer (1932) also suggested that this process would require significant amounts of energy to transport against concentration gradients between the sea and the body fluids and further distinguished the "chloride cells" in the light microscope based on simple morphological characters including large size, a spherical nucleus and staining with eosin. Building on these findings, August Krogh (Krogh, 1939; Krogh, 1938) offered the first idea that transport of ions could be accomplished by independent active transport mechanisms in the gill. Krogh realized that because of the principle of electroneutrality, there would have to be an exchange of counter-ions during efflux or influx of ions across the gill and suggested that sodium (Na^+) uptake was linked to proton (H^+) excretion and Cl^- uptake linked to bicarbonate (HCO_3^-) excretion, a theory still maintained in the current model. However, the exact location, specific types of gill cells and mechanisms for ion transport were unknown at that time and remain the topic of

current research. Development of the electron microscope allowed for ultrastructural description of the ion transporting cells (Doyle and Gorecki, 1961). The "chloride cells", located on the gill filament at the base of the lamellae, were found to be rich in mitochondria, have dense vesicular-tubular network in the cytoplasm and have an ovoid nucleus (Karnaky, 1980; Laurent and Dunel, 1980; Hootman and Philpott, 1978). A large number of mitochondria were predicted for highly active ion transporting cells due to the large ATP requirement of the transport against electrochemical gradients (Krogh, 1938). Definitive physiological proof of salt secretion by the "chloride cells" of marine teleosts waited for almost 50 years from Krogh's initial hypothesis until Foskett and Scheffey (Foskett and Scheffey, 1982), utilizing the vibrating probe technique, localized conductances and Cl^- current to these "chloride cells" in killifish opercular epithelia. This research firmly established "chloride cells" as salt-secretory cells in seawater teleosts.

Further research demonstrated that "chloride cells", or cells with similar morphological characteristics to those described in seawater gill epithelia, were found to also be present in the gills of freshwater fishes (for review see Laurent, 1984). Their function in both systems was first postulated to be in Cl^- transport (Maetz, 1976; Epstein *et al.,* 1973; Krogh, 1938; Keys and Willmer, 1932). However, additional research also suggested multiple functions for the freshwater "chloride cells" including Na^+ transport (Reid *et al.,* 2003; Marshall, 2002; Goss *et al.*, 1992a), Ca^{2+} transport (Li *et al.,* 1997; McCormick *et al.,* 1992; Perry and Flik, 1988) and acid-base regulation (Perry and Flik, 1988; Hobe *et al.,* 1984; Maetz, 1976). Due to the multiple attributed functions of these so called "chloride cells", many authors

have recently began to refer to these cells simply as mitochondria-rich (MR) cells as their function may not be solely for Cl^- transport. Throughout the rest of this thesis, I will use the nomenclature "MR cells" to refer to the mitochondria-rich ion and acid-base transporting cells of the teleost fish gill.

MR Cells in an Analogous Physiological System; the Mammalian Cortical Collecting Duct:

The concept of ionoregulation in the gill cells is similar in theory to the design of other well studied models, including the mammalian cortical collecting duct (CCD) (Turnheim, 1991) and frog skin (Smith, 1971). These systems have a relatively high resistance to paracellular ion permeation, (so-called "tight epithelia") necessary to reduce passive ion loss due to the large gradients that exist across each epithelium. The freshwater fish gill requires a tight epithelium, as the external environment is dilute water (usually < 2 mOsmol/L, $[Na^+] < 0.5$ mM) and the internal surface is in contact with the extra-cellular fluid which has a high concentration of ions (~290 mOsmol/L, $[Na^+]$ ~140 mM). A high resistance epithelia reduces passive ionic loss and reduces the energetic cost of keeping ion concentrations constant.

The mammalian CCD shows striking structural and functional similarities to the fish gill. Some of the functional similarities that exist between the mammalian CCD and the fish gill include apical surfaces that face hypotonic solution (freshwater, dilute urine) and basolateral surfaces face extracellular fluid (ECF), cells that function in uptake of ions and excretion of acid/base equivalents and all these epithelia types form a high resistance membrane to stop passive ion flow (Madsen and Tisher, 1986; Madsen and Tisher, 1985). Structurally, these membranes are also quite similar in

appearance. The fish gill possesses MR cells (~5% of surface area of gill depending on the environment) that are similar in size and appearance to those present in mammalian CCD (termed intercalated cells in the kidney) (Brown *et al.,* 1988). One notable difference between the fish gill and the mammalian CCD that was apparent at the beginning of this study was the functional separation of the MR cells in the mammalian CCD into two physiological sub-types (α- and β-cells) (Furuya *et al.,* 1991; Muto *et al.,* 1990) while definitive physiological characterization of MR cell sub-types in the fish gill was yet to be demonstrated.

The role of the fish gill is largely analogous to the function of the mammalian kidney. Both systems function in primarily in acid-base and ion regulation of the whole animal by differential expression and activity of acid-base relevant ion transporters on the apical membrane of the MR cells (the intercalated cells (IC) in the mammalian CCD) (Goss *et al.,*1992b). While the mammalian CCD is a well studied epithelia, the fish gill is less well characterized, primarily due to structural difficulties associated with the fish gill making it impossible to isolate the epithelium. In the present thesis, the functional similarities that exist between these two epithelia have been used to generate testable hypotheses about function of the ion transporting cells in the fish gill.

As previously mentioned, the MR cells of the mammalian kidney are subdivided into two sub-populations, consisting of the α-IC cells, which have been demonstrated to be linked to acid secretion via an apically located H^+-ATPase, and the β-cells, which are linked to base secretion via an apically located Cl^-/HCO_3^- anion exchanger (AE) (Al Awqati, 1996; Furuya *et al.,* 1991; Muto *et al.,* 1990; Alper *et al.,*

1989; Schuster *et al.*, 1986). The principle of electroneutrality intimately links acid-base regulation with ion uptake (specifically Na^+ and Cl^-) and thus ion regulation can also be attributed to each of the MR sub-types. To maintain electroneutrality, the α-cells have a functional rheogenic coupling of the H^+-ATPase to an epithelial Na^+ channel (Turnheim, 1991) while β-cells move Cl^- as the counter-ion through the apical AE (Stetson and Steinmetz, 1985).

At the beginning of this thesis, the fish gill model was much less well developed than many other epithelial models. The presence of an apical Cl^-/HCO_3^- exchanger for ion and acid-base regulation was well established (Claiborne *et al.*, 2002; Hyde and Perry, 1989; Wood *et al.*, 1984) while the apical Na^+ uptake was thought to be accomplished via H^+-ATPase rheogenically coupled to a Na^+ channel (Claiborne *et al.*, 2002; Perry, 1997; Avella and Bornancin, 1989). However, the molecular identity and localization of each of these transport processes remained to be determined. Importantly, the fish gill model presented these two processes on a single cell type (see Figure 1.1) while for most other epithelia studied, functional separation of base (HCO_3^-) and acid (H^+-ATPase) excretion occurred in two separate cell types, the α- and β-IC cell of the mammalian CCD, respectively can be used as a prime example. At the beginning of this study, the models for fish gill and mammalian kidney were as follows:

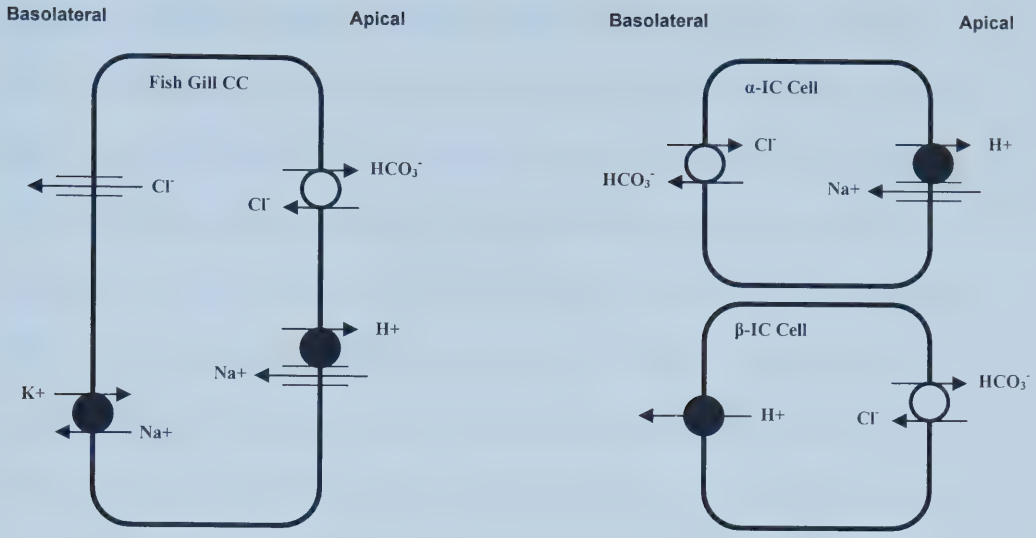

Figure 1.1

Early models of fish gill "chloride cell" and mammalian kidney IC cells showing some of the common transport processes, with particular attention to acid/base regulation.

Despite the fact that all of the transport processes required for acid-base balance and ion regulation are known to occur in the fish gill, there was no apparent success in physiologically characterizing different sub-populations of MR cells in the fish gill. The idea that an analogous system, such as the mammalian kidney, could be used to generate hypotheses based on structure/function relationships with the fish gill is common practice in comparative physiology and was also used to develop testable hypotheses and methodologies for furthering our understanding of the fish gill.

It was reported that sub-types of MR IC cells of the CCD could be identified based on binding of the peanut lectin agglutinin (PNA) (Schuster *et al.*, 1986). Only one type of IC cell (β-cell) was found to bind the peanut lectin on the apical surface,

yielding a method to distinguish these two separate functional cell types (Satlin *et al.*, 1992). This method of distinguishing IC cells has recently been used to separate IC cells from rabbits, based on PNA binding and fluorescent-activated cell sorting (FACS), to obtain a pure population of β-type IC cells. This pure population of IC cells was then used to clone a new member of the AE and Na^+/HCO_3^- cotransporter (NBC) superfamily, called AE4 (Tsuganezawa *et al.*, 2001). This paper provides evidence that the PNA binding separation technique can be very useful for distinguishing pure populations of β-type IC cells. Given the similarity between the IC cell of the kidney cells and the gill MR cells in both structure and function, I wanted to explore the possibility of separating the MR cells in the gill to identify if there were different cell types present in the fish gill and to functionally characterize each of these cell types.

Evidence for Sub-types of MR Cells in the Fish Gill:

Numerous investigations have advanced the understanding of these MR cells in both seawater and in freshwater by examining the ultrastructure and making comparisons between many species (Perry, 1997). The ultrastructure of these MR cells is very well conserved throughout the different species studied (Pisam *et al.*, 1990; Doyle and Gorecki, 1961). Physiologically, understanding MR cell function has lagged with measurement of Na^+/K^+-ATPase activity the only apparent functional test for the presence and/or activity of MR cells in whole gills. Na^+/K^+-ATPase activity is known to increase in concert with increases in Na^+ and Cl^- uptake in freshwater and also during adaptation to seawater (Mancera and McCormick, 2000). However, while the ultrastructure of the MR cells is similar between species, some

researchers have suggested that there exist sub-types of the MR cells to perform different functions based solely on small differences in ultrastructure. Transmission electron microscopy (TEM), using osmium staining, has shown that there are darker and lighter forms or MR cells in the fish gill, based on electron density (Doyle and Gorecki, 1961). These dark and light forms existed in all fish regardless of the water medium they come from (*i.e.* freshwater or seawater). Further work on this sub-typing of the MR cell led to nomenclature proposed by Pisam and co-workers (1987) calling these cells α- and β-cells (lighter and darker, respectively). These α- and β-cells are separated by morphological characters such as shape, apical surface and location in the gill. The α-type cells are located on the base of the lamella, while the β-type cells are found in the inter-lamellar regions. Pisam and colleagues (Pisam *et al.,* 1987) showed that during seawater transfer there was a transition from the two types (α and β) in freshwater environments to only one seawater adapted MR cell, consisting of the α-type MR cells (Pisam *et al.,* 1987). More recently, Wong and Chan (1999) used flow cytometry to track the change in MR gill cell populations during seawater transfer using parameters of relative cell size, autofluorescence and granularity. They demonstrated that in freshwater there exists two populations of MR cells and upon transfer to seawater, only one population appears to be present. Taken together, these studies suggest existence of different MR cell sub-types and alteration of function and abundance during seawater adaptation. They suggested that the sub-types perform different ionoregulatory functions and the relative proportion of each type depends on the environment the fish is exposed to (*e.g.* freshwater vs. seawater).

However, a significant drawback of all these earlier studies was that there was no evidence for functional sub-types of MR cells.

For many years it was believed that the uptake of Na^+ from the water and the excretion of H^+ was through a sodium/proton exchanger (NHE) located on the apical membrane of gill cells as initially proposed by Krogh (1939). Similarly, Cl^- uptake from the water was also hypothesized to occur via an apical Cl^-/HCO_3^- exchanger. These transport mechanisms were thought to occur on the same cell type as excellent correlations could be generated between unidirectional uptake in both Na^+ and Cl^- and exposed area of MR cells (by SEM) (Perry *et al.*, 1992; Perry and Laurent, 1989; Wood *et al.*, 1984). A series of experiments looking at morphological change and uptake kinetics of Na^+ and Cl^- with rainbow trout (Goss and Perry, 1993) demonstrated these relationships for Na^+ and Cl^- uptake were the same, regardless of the habitat of the fish (brown bullhead is a benthic species and rainbow trout are pelagic euryhaline).

However, with proposed sub-types of MR cells based solely on morphological characters, theories were formulated about the Na^+ and Cl^- movement being attributed to different cell types (Lin and Randall, 1991; Avella and Bornancin, 1989). Furthermore, the model of NHE was suggested to be thermodynamically impossible in very dilute freshwater and the NHE mechanism was replaced by a H^+-ATPase coupled electrochemically with a Na^+ channel (Avella and Bornancin, 1989). The probable location for these transporters linked Na^+ to the pavement cells and Cl^- regulation to the "chloride cells". The first evidence for dissociation of Na^+ and Cl^- unidirectional flux was provided by Goss *et al.* (1992a) in hypercapnic (high P_{CO2})

brown bullheads. The acid/base disturbance imposed by hypercapnia removes the co-dependence of Na^+ and Cl^- fluxes that is present in normal steady-state conditions and allows independent examination of the location and function of the transporter. Specifically, they demonstrated that Cl^- movement during acid/base disturbance was correlated strongly with MR cell surface area (demonstrated by SEM), but no correlation could be described for Na^+ transport suggesting that Na^+ transport does not occur via the MR cells. Further, they demonstrated that anion exchange (Cl^- /HCO_3^- exchange) in the "chloride cells" could be controlled by physical alteration of the gill epithelia. The fractional area of the "chloride cells" could be reduced via covering by adjacent pavement cells, thus reducing the exposed area of the "chloride cells" to the environment and rate of anion exchange. There was a direct correlation of increased exposed area of "chloride cells" with Cl^- uptake. The pavement cells also underwent morphological changes (microvillar density) that coincided with the acidosis and increased acid excretion. The changes in the pavement cells suggest a link in Na^+ influx and H^+ secretion, supporting the theory of Na^+ uptake driven by H^+ secretion on a separate cell type.

At this time, most research programs focused on proving/disproving the H^+-ATPase/Na^+ channel model as no technology existed for direct examination of MR cell subtypes. Immunolocalization of the H^+-ATPase has yielded apparent conflicting results. Sullivan *et al.* (1995) have shown that the protein is located primarily in the apical membrane of lamellar (presumably pavement) cells and this expression increases appropriately during hypercapnia. In contrast, Wilson *et al.* (2000) have shown that the H^+-ATPase is present in both "chloride cells" and pavement cells.

However, use of different antibodies and strains of fish makes resolution of the apparent conflict difficult to resolve.

Involvement of MR Cells in Toxicology

Metal toxicity is a serious problem to fish as many metals have been shown to interfere with ion transport, such as Ag and copper (Cu), which inhibit Na^+ and Cl^- transport (Morgan *et al.,* 1997) and divalent heavy metals (cadmium, zinc and cobalt) which interfere with calcium (Ca^{2+}) transport (Hogstrand *et al.,* 1996; Verbost *et al.,* 1987). These metals can block or interfere with normal physiological uptake mechanisms (such as Na^+ channel in the case of Ag^+), or by binding to the negative charges found on the apical surface of cells causing membrane instability or disruption. The transport of ions, particularly Na^+ and Cl^- as described previously, is by the MR cells in the fish gill. A keen area of research has been how these metals from the environment get into the fish through the MR cells and exert their effects.

A method used to examine metals uptake, as well as ion transport, has been to use isotopes to follow uptake of the ion of interest. Ion transport has been evaluated by using isotopes of Na^+ and Cl^- to study ion regulation in fish starting as early as 1956 (Maetz, 1956). Metal isotopes can be used in the same fashion as Na^+ and Cl^- isotopes to determine rates and location of uptake within an animal. Our initial research using the PNA cell types and isotopes has shown that the PNA^- (non PNA binding) cells are responsible for Na^+ transport, so we can predict that metals affecting Na^+ transport (such as Ag or Cu) should show affects on ion uptake within the PNA^- cells.

Research conducted by Bury and Wood (1999) has explored the possibility that Ag^+ may enter through the epithelial Na^+ (ENaC type) channel on the apical surface of the fish gill. They have shown a phenamil-sensitive (specific ENaC blocker) reduction (78%) in Ag^+ uptake in whole body rainbow trout, which supports the freshwater model of Na^+ uptake as presented in Figure 5.1 and gives evidence for entry of Ag^+ through the Na^+ channel. These authors also demonstrated a reduction in Ag^+ uptake using bafilomycin (baf), a H^+-ATPase blocker. This result would be predicted by our freshwater model (see fig 1.1)(ENaC linked to H^+-ATPase), as it is the H^+-ATPase that creates the electrical driving force for rheogenic entry of Na^+. The toxicity of Ag^+ up to this point has not been attributed to a specific type of MR cell in the gill. The mechanism of uptake is generally believed to be through the apical Na^+ channel, but which cell type is responsible for the uptake of Ag^+ has not yet been described. Given the current gill cell model, and our previous research attributing acid-inducible, phenamil sensitive Na^+ influx to the PNA^- cells (Reid *et al.*, 2003), the most probable cell type responsible for the uptake of Ag^+ will be the PNA^- MR gill cell.

Objectives of Research

With the abovementioned literature in mind, I developed a series of successive hypotheses relating to the existence, characterization and importance of specific MR cells to fish physiology and toxicology. The primary objectives of the thesis are:

1. To demonstrate that there exist two sub-types of MR cells in freshwater rainbow trout gills.

2. To examine the changes in the relative distribution of PNA^+ and PNA^- cell types during transfer from a freshwater to a seawater environment.
3. To examine changes in the functional properties (rates of activity, expression of transport proteins etc.) of both PNA^+ and PNA^- cell types during environmental stress (such as seawater acclimation).
4. To test whether the mechanism of entry of the toxic metal silver (Ag^+) is via permeation through the identified apical Na^+ channel in freshwater fish.

The first stages of this thesis demonstrate that the fish gill possesses sub-populations of MR cells that differentially bind PNA, termed PNA^+ (binding) and PNA^- and that they are functionally distinct cell populations. To date, functional analysis of this PNA^+ cell type has been somewhat difficult due to lack of specific inhibitors for anion channels, but we have been able to identify Na^+ uptake in the PNA^- MR cells (Reid *et al.*, 2003). The results presented in Chapter 2 represent my contributions to a published series of manuscripts where I am a co-author (Reid *et al.*, 2003; Galvez *et al.*, 2002) and describe the initial characterization of MR cell sub-types and the technique we developed for separating and characterizing these MR sub-types. I had a fully collaborative role in each of these projects. Chapter 3 examines the changes in relative distribution and function of MR cells during seawater adaptation while Chapter 4 examines the mechanisms of Ag^+ toxicity and its affects on Na^+ transport. Chapters 3 and 4 represent research designed and performed by me. The ability to be able to separate the gill cells by the magnetic cell separation

method that we have developed is a distinct advantage in that we can now assess the functional characteristics of these cell types and have a greater understanding of the transport physiology involved in different MR cell populations.

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Chapter 2: Characterization of Sub-types of MR Cells in Freshwater Rainbow Trout.

Introduction:

The gill epithelium of fish is the site of acid/base and ion regulation. The MR cells of the gill have large quantities of mitochondria to produce the energy (ATP) required for this regulation. Recently, the model of ion regulation has linked Cl^- uptake with base extrusion and Na^+ uptake with H^+ extrusion (review by Perry, 1997). Cl^- uptake is thought to occur through an apical anion exchanger and Na^+ uptake via an apical channel linked electrogenically to an H^+-ATPase. The precise location or molecular identity of the proteins involved in these transport processes is largely still unknown.

The gill epithelium is composed of many different cell types and the transport of ions was originally thought to occur through the "chloride cells" (Karnaky *et al.*, 1976; Maetz, 1976). Early work identified these cells as the ion transporting cells based on characters associated with transporting cells, such as high mitochondrion numbers, well developed vesicotubular network and high levels of Na^+/K^+-ATPase expression (Karnaky, 1980; Hootman and Philpott, 1978). The "chloride cell" was believed to be the ion transporting cell for many years, until Goss *et al.* (Goss *et al.*, 1994; Goss *et al.*, 1992) conducted experiments to show that "chloride cell" fractional area was correlated to Cl^- uptake, but not to Na^+, in fish with acid-base disturbances. This was the first evidence that Na^+ transport could not be accounted for by the

"chloride cells". Other researchers have shown Na^+ transport to be associated with the pavement cells of the gill. Sullivan *et al.* (1995) showed H^+-ATPase to be associated with the apical region of lamellar pavement cells, using immunohistochemistry, and that the expression of the H^+-ATPase increased with hypercapnia (acidosis). Expression of the H^+-ATPase was unaffected by alkalosis, supporting the thought that HCO_3^- extrusion was not linked to these cell types.

Prior to the start of this study, our lab had demonstrated that there are at least two distinct sub-populations of MR gill cells in the rainbow trout based on differential binding to PNA (Goss *et al.,* 2001). Functional identification of MR cell sub-types had previously been demonstrated in the intercalated cells of the mammalian CCD. PNA was shown to binds specifically to an external marker located exclusively on the β-IC of the mammalian kidney (Satlin *et al.,* 1992). However, at the start of this study, there had been no physiological or biochemical evidence for distinct populations of MR cells in the gill. Other research had indeed proposed that there are different MR cell subtypes in fishes, but the work was based solely on morphological characters, such as ultrastructural differences and changes in autofluorescence (Wong and Chan, 1999; Pisam *et al.,* 1987). The aims of this study are to develop a technique to isolate and characterize sub-populations of MR cells. TEM was used to morphologically identify these sub-populations and Western blotting was used to characterize differences in functional proteins during acid/base disturbances. Radioactive flux analysis, using $^{22}Na^+$, was also used to determine which of these sub-populations of MR cells were responsible for Na^+ transport.

Methods:

Experimental animals

Adult rainbow trout (~200 g) (*Oncorhynchus mykiss*) from Alberta Trout Growers were maintained in flow-through 450 L fiberglass tanks supplied with aerated dechlorinated Edmonton tap water (hardness 160 ppm as $CaCO_3$, total alkalinity 120 mg/L; pH 8.2). Water temperature was maintained at 15°C, and the photoperiod matched the natural regime of Edmonton, Alberta, Canada. Fish were fed once daily with dry trout pellets.

For experiments to develop the gill cell isolation and magnetic separation protocols, fish were randomly selected from holding tanks and killed immediately by immersion overdose (1 g/L MS-222, Syndel) followed by a cephalic blow. Fish used for the hypercapnia or base infusion experiments were removed from holding facilities and placed individually in darkened, aerated flow-through Plexiglas aquariums (holding boxes with ~6 L capacity). Fish were allowed to acclimate to the aquariums for at least 24 h before experimentation. To induce an acidosis in the fish, in-flowing water was switched to water rendered hypercapnic by bubbling with 1% CO_2 in air. In addition, holding boxes were individually aerated with 1% CO_2 in air. Control parallel experiments were run with fish maintained under normocapnic conditions. Mixing of gas for aeration of boxes and in-flowing water was accomplished using 100% CO_2 (Praxair) and compressed air (1:99) using calibrated flow meters and a gas equilibration column. To induce an alkalosis in the fish, a base-infusion protocol similar to that of (Goss and Wood, 1990) was followed. Briefly, fish were lightly anesthetized with 0.1 g/L MS-222 and implanted with

polyethylene cannulas (PE-50; Clay-Adams) into the dorsal aorta. Fish were allowed to recover from surgery for 48 h and then were infused for 24 h with either $NaHCO_3$ (140 mM, pH 7.8) or, as a parallel control, NaCl (140 mM, pH 7.8) at a nominal rate of ~1000 μmol/kg body wt/h.

Materials

Streptavidin-conjugated Alexa fluor 594, Mitotracker Green-FM and calcein-AM were obtained from Molecular Probes, Eugene, OR. The magnetic cell separation system and anti-FITC microbeads used for MACS separation were from Miltynei Biotech (Auburn, CA). Antibodies to Sco1, a yeast mitochondrial integral membrane protein, were obtained from Dr. M. Glerum, University of Alberta. Antibodies to the 31-kDa subunit of the kidney V-type H^+- ATPase were obtained from Dr J. N. Fryer, University of Ottawa. Antibodies against the α-subunit of the Na^+/K^+-ATPase (*Drosophila*) were obtained from Dr. Doug Fambrough, Johns Hopkins University. Horseradish-peroxidase-coupled goat anti-rabbit secondary antibody and Luminol chemiluminesence detection reagents were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Commercial BCA protein kit was obtained from Pierce (Rockford, IL). All other reagents used in the protocol were obtained from Sigma Chemicals, St. Louis, MO.

Gill digestion protocol

Gill arches were excised from the fish, rinsed in dechlorinated tap water to remove coagulated blood and mucus, and lightly blotted to remove excess water. Gill

filaments were cut from the gill rakers and placed in ice-cold Cortland's saline (in mM: 143 NaCl, 5.0 KCl, 1.5 $CaCl_2$,1.0 $MgSO_4$, 5.0 $NaHCO_3$, 3.0 NaH_2PO_4, 5.0 glucose; pH 7.8). Gills were digested in 0.2 mg/mL collagenase (type 1A) in Cortland's saline for 20 min at 18°C with continuous circular agitation (300 rpm). Gill filaments were scraped with a glass microscope slide, and the gill suspension was filtered through 254- and 96-μm nylon meshes to remove large debris. The final filtrate of dispersed cells was diluted in PBS (in mM: 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, 1.4 NaH_2PO_4; pH 7.8) and centrifuged at 500 *g* for 10 min at 4°C. The absence of Ca^{2+} from the media significantly reduced clumping and aggregation of dispersed cells during subsequent steps of the isolation protocol. Dispersed cells were washed twice with at least 10 vol of PBS and resuspended in 2 mL PBS, placed on a 1.09 g/mL Percoll solution, and spun for 10 min at 500 *g* (4°C) to separate contaminating red blood cells (RBCs) from the gill cell suspension. The gill cells remained on top of the 1.09 g/mL Percoll solution, whereas RBCs pelleted to the bottom of the tube. Gill cells were again collected, diluted in ~10 vol of PBS, centrifuged for 10 min at 500*g*, resuspended in 2 mL PBS, layered over either a two-step gradient consisting of 1.03 and 1.09 g/mL Percoll or a three-step gradient consisting of 1.03, 1.06, and 1.09 g/mL Percoll (4 mL each) and centrifuged at 2000 *g* (45 min). Consistent with the findings of Goss *et al.* (2001), distinct bands of cells were collected from the Percoll interfaces of the discontinuous gradients after centrifugation. Cells from each of the Percoll interfaces were collected independently, washed once in ~10 vol PBS, centrifuged (2000 *g*; 10 min), resuspended in 0.5–2.0 mL PBS, and used for analysis as appropriate. Gill cells from

the 1.06 – 1.09 g/mL Percoll interface (MR cells) of the three-step gradient were further separated into PNA^+ and PNA^- fractions using MACS as outlined below.

Fluorescence microscopy and fluorescence spectrophotometry

Dispersed gill cells (1.03 – 1.06 g/mL or 1.06 – 1.09 g/mL) were incubated in PNA-FITC in PBS (20 min; 40 μg/mL) to identify and quantify the PNA^+ cells using fluorescence microscopy. Mitotracker green-FM (20 min; 100 nM) was used to characterize mitochondria staining in the different fractions of isolated dispersed cells using both an inverted microscope, to identify staining in single cells, and a fluorescence plate reader, to quantify mitochondria staining in gill cell populations. The percentage of MR cells binding PNA was determined by labeling cells first in biotin-conjugated PNA (PNA-biotin; 20 min, 40 μg/mL), washing the cells, and then double labeling the cells with streptavidin-Alexa fluor 594 (20 min; 20 μL/mL) and Mitotracker green-FM (20 min; 100 nM). Gill cells were washed twice in PBS to remove unbound dye(s). For fluorescence microscopy, cells were placed on glass slides for differential interference contrast (DIC) microscopy (Nikon Eclipse 300) and fluorescence imaging (TE-FM Epi-Fluorescence attachment) using an inverted microscope. The fluorescence microscope was equipped with epi-illumination via a Xenon arc lamp (Lambda LS, Sutter Instruments, Novato, CA). PNA-FITC, Mitotracker green-FM or Calcein-AM (excitation 495 ± 5 nm; dichroic 515 nm cut on; emission 540 ± 25 nm) and PNA-Alexa fluor 594 were imaged (excitation 560 ± 20 nm; dichroic 595 nm long pass; emission 630 ± 30 nm) using Plan-Fluor objectives at ×40 [0.6 numerical aperture (NA) air] and ×100 (1.3 NA oil

immersion). Images were digitally captured on a 12-bit charge-coupled device camera (Cooke SensiCam, Kelheim, Germany). Fluorescence images were often binned at 2 × 2 to increase the sensitivity of the fluorescence capture. Final images were adjusted for contrast and brightness only using Adobe Photoshop 6 software. The difference in fluorescence between the MR and non-MR cells was blatant enough that the adjustment of contrast had no adverse affect on the identification of MR cells in the images. To examine relative mitochondrial staining in the fractions, 20×10^3 Mitotracker-labeled cells in 200 µL PBS (as above) from each fraction were loaded into a 96-well plate and relative fluorescence intensity was read (excitation 485 ± 25 nm; emission 538 ± 25 nm) using a fluorescence microplate spectrophotometer (Molecular Dynamics fmax).

Magnetic cell separation protocol

Magnetic cell separation was used to isolate PNA^+ cells. Recent studies in our laboratory (Goss *et al.*, 2001) showed that PNA^+ cells fractionate exclusively to the 1.06 – 1.09 g/mL Percoll interface and magnetic separation was only performed on cells from this fraction. Gill cells collected from the 1.06 – 1.09 g/mL Percoll interface were incubated in PNA-FITC and rotated continuously. Cells were incubated in a solution of anti-FITC antibodies covalently coupled to 50 nm iron particles (10 µL anti-FITC microbeads/mL PBS) for 20 min at 4°C with continuous mixing. Cells were washed once to remove unbound anti-FITC microbeads and resuspended in 1 – 2 mL of degassed PBS buffer. The gill suspension was passed through a 30-µm filter attached to the top of a positive selection iron column (LS^+,

Miltylnei held within a magnetic field). Cells with PNA-FITC and anti-FITC microbeads bound would be retained in the column as long as the magnetic field was present. The column was given 3 × 3 mL rinses with PBS. The cells passing through the column in the presence of the magnetic field were collected and termed the PNA^- fraction. The column was then removed from the magnet and 5 mL PBS was added to the column and the cells were eluted from the column using a plunger. The eluted cells were termed the PNA^+ fraction. Both fractions were centrifuged at 500 *g* for 10 min at 4°C. The supernatant was aspirated, and the cell pellet was resuspended in 0.5 – 1.0 mL PBS buffer.

Western blot analysis

Samples collected from each of the fractions were placed in denaturing buffer (500 mM Tris-HCl, 5% glycerol, 10% SDS, 1.0% bromophenol blue, 5.0% mercaptoethanol) and analyzed by SDS-PAGE (10% acrylamide) followed by semi-dry horizontal transfer (Bio-Rad) onto nitrocellulose membranes for immunoblotting. To ensure equivalent loading in each lane, equal cell numbers (Sco1 experiments) or equivalent total protein (for analysis of Na^+/K^+-ATPase and H^+-ATPase expression) was loaded into each lane. After transfer, nitrocellulose sheets were placed in blocking buffer (5% skim milk powder in antibody buffer-140 mM NaCl, 10 mM Tris base, 0.03% Tween 20, pH 7.4) and incubated for 1 h at room temperature. After washing with antibody buffer (3 × 20 min), blots were incubated overnight (4°C) on a rocking platform in antibody buffer plus 1% skim milk powder containing antibodies raised against Sco1 (1:1,000), anti Na^+/K^+-ATPase (1:2,500), or anti V-type H^+-

ATPase 31-kDa subunit (1:2,500). The following morning, blots were quickly rinsed in double-distilled H_2O and washed in large volumes of antibody buffer (3 × 20 min). The secondary antibody, HRP-conjugated anti-rabbit IgG (1:5,000 in antibody buffer with 1% skim milk powder), was incubated with the blot on a rocking platform for 1 h at room temperature. The secondary antibody was removed by washing in antibody buffer (3 × 20 min). Detection of proteins was accomplished using a Luminol Western blotting detection kit (Santa Cruz) and detection on X-ray film. Resultant bands were quantified by densitometric analysis using Scion Image software.

Na^+ influx experiments

Accumulation of Na^+ by isolated trout gill epithelial cells was based on the uptake of $^{22}Na^+$ by a fixed number of cells for a fixed period of time. The cell number and flux time were determined based on initial time course experiments using crude suspensions of gill epithelial cells from a two-step (1.03 – 1.09 g/mL) discontinuous Percoll gradient. To measure $^{22}Na^+$ uptake in cells from each fraction, aliquots were centrifuged (1000 *g*, 5 min) and the pellet resuspended in 1 mL flux solution (pH 7.8, 290 mosmol/L, in mM:157.5 *N*-methyl-D-glucamine (NMDG)-Cl, 5 K-gluconate, 1.5 Ca-gluconate, 1 Mg-gluconate, 5 Hepes (Na free), 1 Na-nitrate) containing the desired combinations of inhibitors. Once re-suspended, 37 kBq $^{22}Na^+$ (1 µL $^{22}Na^+$ stock; NEN, Boston, MA, USA) was added to the wall of the centrifuge tube above the cell suspension and the suspension was mixed. After exactly 1 min, 200 mL (×3) of the cell suspension was removed and placed onto pre-wetted individual glass microfiber filter circles (Whatman GF/C, 2.4 mm diameter, 0.2 mm

pore size) supported by small pieces of plastic mesh, contained within the barrel of 20 mL syringes connected to a vacuum line. The incubation solution was immediately removed by vacuum and the trapped cells rinsed (35 mL) with 154 mM NaCl saline. Preliminary tests of adding an aliquot of $^{22}Na^+$ in the absence of cells demonstrated that 25 mL rinses of the glass microfiber filters were sufficient to reduce $^{22}Na^+$ activity to background levels. This filter-vacuum system allowed for rapid (~10 s) washing of the cells and the removal of free $^{22}Na^+$. In parallel, two 100 mL aliquots of incubation saline were dispensed into individual 7 mL scintillation vials for later determination of specific activity. Washed filter disks were placed in 7mL scintillation vials and 5 mL scintillation cocktail (ACS; Amersham, Baie d'Urfe, QC, Canada) was added. $^{22}Na^+$ activity (c.p.m.) was determined (Beckman LC6200) and corrected for background activity (pre-washed filters in 5 mL scintillation cocktail). To determine the mechanism contributing to overall Na^+ uptake in the isolated cell populations, the flux saline for all conditions contained 50 mM ouabain to remove nonspecific Na^+ efflux from cells due to the action of the Na^+/K^+-ATPase, 20 mM bumetanide to block the $Na^+/K^+/2Cl^-$ (NKCC) co-transporter and 50 mM HOE-694 to block the endogenous basolateral NHE. Amiloride and other amiloride analogues could not be used to block the NHE due to their efficacy at also blocking ENaC family members. HOE-694 (10 mM) is a selective NHE-1 inhibitor without effect on ENaC in mammalian systems (Loh *et al.,* 1996; Woll *et al.,* 1993).

Intracellular acidification and activation of the H^+ excretion/Na^+ uptake mechanism in the isolated cells was accomplished by replacement of NaCl with Na-propionate (kept external [Na^+] at a constant 15 mM). The involvement of H^+-

ATPase in $^{22}Na^{+}$ influx was determined by prior (1 min) incubation of the cells with bafilomycin (10 nM). The involvement of the purported fish gill ENaC homologue in $^{22}Na^{+}$ uptake was determined by prior (1 min) incubation of the cells with the Na^{+} channel blocker phenamil (10 mM).

Calculations

Na^{+} influx (J_{in}^{Na}; measured in nmol $Na^{+}/10^{6}$ cells) was calculated using the following equation:

$$J_{in}^{Na} = (SA \times \text{sample counts}) / \text{cell numbers}$$

where sample counts represents the c.p.m. of individual aliquots of washed cells trapped on the glass filters, SA represents the specific activity of the incubation saline of individual samples (expressed as nmol Na^{+}/c.p.m.), and cell number represents the number of gill cells in the individual aliquots. The calculated Na^{+} influx was expressed as a function of a standard number of cells (1×10^{6}). Na^{+} influx rate was calculated using equation 1 divided by the duration of the incubation and expressed as nmol Na^{+}/min/10^{6} cells.

Statistical analysis

One-way ANOVA and the post hoc Student-Newman-Keuls test were performed using SPSS version 8.0 software to test for significant differences between

control and experimental groups. $P < 0.05$ was used as the fiducial limit of significance.

Results:

To determine if PNA bound to MR gill cells, we used a discontinuous Percoll density gradient to separate and enrich the MR gill cell population (1.06 – 1.09 g/mL Percoll interface). To determine if this population was in fact a MR cell population, we stained the cells with Mitotracker Green-FM (100 nM, 20 min), a mitochondrial stain and viewed the cells under fluorescence microscopy. It was determined that almost all of the cells isolated using the Percoll density gradient were MR cells (Figure 2.1 A and B). In addition to this, we tested to see if these MR gill cells would bind PNA. The MR cells were incubated with PNA- FITC (40 μg/mL, 15 min) for viewing with fluorescence microscopy. It was demonstrated that a sub-population of the MR cells bound the PNA (Figure 2.1 D) and were termed PNA^+ cells. These results demonstrate that there are at least two distinct sub-populations of MR gill cells in the trout, termed PNA^+ and PNA^- (Figure 2.1 F).

When the MR cells were isolated and incubated with PNA, we wanted to determine the relative percentages of MR cells that were PNA^+ and PNA^-. Trout from freshwater were used with discontinuous Percoll gradient centrifugation to collect the MR gill cells, incubated with PNA-Alexa Fluor 594 and Mitotracker, and viewed under fluorescence microscopy to count the number of cells that were both mitochondrial rich and bound PNA (PNA^+ MR cells). A DIC image was used to count the total number of cells in the field of view of the microscope. A fluorescence image was then captured to determine which of the cells in the field of view were mitochondrial rich (stained positive for Mitotracker). Lastly, a fluorescence image of the cells was captured to look at how many of the MR cells were positive for PNA-

Alexa Fluor 594 (PNA^+). Only the MR cells were found to bind PNA and we used a minimum of 5 randomly chosen fields of view for each fish. We demonstrated that in a freshwater trout 64.4 ± 3.8 % of the MR cells were found to be PNA^- and 35.6 ± 3.8 % of the MR cells were PNA^+ (Figure 2.2).

Quantitative analysis of the mitochondrial content of each of the gill cell fractions was performed using Mitotracker and a fluorescence plate reader. Gill cells from the 1.06 - 1.09 g/mL Percoll interface showed a significant (~ 4-fold) increase in mitochondrial fluorescence over the 1.03 - 1.06 g/mL fraction (Figure 2.3). When the MR cells from the 1.06 - 1.09 g/mL fraction were further separated into PNA^- and PNA^+ cells using MACS, we saw a significant difference between the cell types, with the PNA^+ showing a higher relative fluorescence than the PNA^- cells (Figure 2.3). Both the PNA^- and PNA^+ cell types showed significantly higher mitochondrial fluorescence than the 1.03 - 1.06 g/mL fraction that represents the pavement cell type. To further validate the findings that both of the PNA sub-types are indeed mitochondrial rich, we performed Western blotting of the fractions using an antibody to the inner mitochondrial membrane protein (Sco1), a molecule with a high degree of molecular conservation across numerous phyla. This antibody was raised against yeast Sco1, but we demonstrated heterologous cross reactivity for fish gill. Western blot analysis was performed on all Percoll fractions. The 1.06 - 1.09 g/mL fraction was also separated further into PNA^- and PNA^+ sub-types for analysis. Sco1 levels in the PNA^- and PNA^+ cell fractions were 23- to 26-fold higher than in the 1.00 - 1.03 g/mL fraction (comprised of mostly mucous cells) and ~4.5-fold higher than in the 1.03 - 1.06 g/mL fraction (Figure 2.4), which correlates very well with the data

presented in Figure 2.3. However, we did not observe a difference between the PNA sub-types using Sco1, suggesting that both isolated sub-types are mitochondria rich.

We hypothesized that the PNA-differentiated MR cell sub-types were functionally distinct populations and should respond independently during environmental stress, much like the MR cell sub-types in the mammalian cortical collecting duct (Al-Awqati, 1998). To test this hypothesis we exposed fish to hypercapnic (acidotic) conditions (1% CO_2 for 24 hr) and alkalotic conditions (continuous $NaHCO_3$ infusion) and examined, by Western blotting and densitometry, and the changes in Na^+/K^+-ATPase expression during the experimental stress (Figure 2.6). All lanes were loaded with equivalent amounts of total protein and data were expressed as the ratio of PNA^- to PNA^+ for each fish. Changes in Na^+/K^+-ATPase activity due to increased numbers of one cell type over another are removed in this way. Relative Na^+/K^+-ATPase expression in untreated fish was only ~0.3, indicating that under control conditions, the PNA^+ cells show about 3-fold higher expression than PNA^- cells (Figure 2.6). Acidosis, caused by hypercapnia, caused a 3-fold increase in expression of Na^+/K^+-ATPase, suggesting either an increase in the number of PNA^- cells, or decreased expression in the PNA^+ cell fraction. During alkalosis, the expression of Na^+/K^+-ATPase also increased (~2.5-fold) in the PNA^- cells (Figure 2.6).

We used the same treatments to examine relative expressions of H^+-ATPase in the gill cell fractions using an antibody that was previously shown to demonstrate H^+-ATPase expression levels in the gills of rainbow trout (Sullivan *et al.*, 1995). The expression of H^+-ATPase was found to be ~2-fold higher in PNA^- than PNA^+ cells

under control conditions (Figure 2.5). We also saw an increase in this expression (~60%) with hypercapnia showing that the expression of the H^+-ATPase was increased in the PNA^- cells over the PNA^+ cells during acidosis (Figure 2.5). Both the $NaHCO_3$ and the NaCl treated fish showed no significant difference from the untreated control fish.

The PNA MR populations of gill cells were used to examine independent radioisotope fluxes with $^{22}Na^+$. We hypothesized that the PNA^- cell type should be responsible for Na^+ transport via an apical Na^+ channel linked to an H^+-ATPase, much like the α-IC cells of the mammalian kidney. Radioisotope flux analysis was performed with various blocking drugs to inhibit any Na^+ transport sites other than the Na^+ channel. Also, we used propionic acid to cause internal acidification in the cells to help drive uptake of $^{22}Na^+$ through the apical Na^+ channel. Our analysis demonstrates that in the PNA^+ cells there is no acid inducible Na^+ transport (Figure 2.7). However, in the PNA^- cells we do see an acid inducible Na^+ transport that can be blocked by the addition of phenamil (Na^+ channel blocker), indicating the presence of phenamil sensitive Na^+ uptake in the PNA^- cells, but not in the PNA^+ cells (Figure 2.7).

Discussion:

In this present study we have developed and utilized a novel magnetic bead separating system that is able to take advantage of the binding of PNA to a sub-type of MR cell from the freshwater fish gill. PNA is a lectin that binds specifically to the terminal D-galactosyl residues of specific glycosylated proteins (Lotan *et al.,* 1975). This particular residue is rare, and thus can be used as a specific cellular marker when identified. This binding of PNA to the MR cells sub-population has been demonstrated by earlier work in our lab (Goss *et al.,* 2001). The cells that bind the PNA (PNA^+) have ultrastructure similar to the classic "chloride cells" (Galvez *et al.,* 2002). The PNA^- fraction has an ultrastructure more similar to the pavement cells, but has a high number of mitochondria (not normally a characteristic of pavement cells). We have used Western blot analysis to examine changes in protein expression of the Na^+/K^+- and H^+-ATPase in the MR cells during respiratory acidosis and metabolic alkalosis.

Sub-types of MR cells have been reported in previous work on fishes (Pisam *et al.,* 1995; Pisam *et al.,* 1990) based on differences in ultrastructure and staining characteristics of electrophilic stains used in TEM analysis. Pisam and colleagues termed these cell sub-types as α- and β-cells. These cells were both present in freshwater fish, but the β-cells disappeared upon adaptation to seawater. More recently, Wong and Chan (1999), using discontinuous density centrifugation and flow cytometry, identified two types of MR cells in the gills of freshwater adapted Japanese eels. Our lab has also shown that the MR cells from the gill of the rainbow

trout also migrate to a specific (1.06 – 1.09 g/mL) discontinuous Percoll interface (Goss *et al.,* 2001), similar to Wong and Chan.

Sub-typing of MR cells in the kidney is often accomplished by binding of PNA to the apical surfaces of some of these cells (Al-Awqati *et al.,* 1998; Satlin *et al.,* 1992). The binding of PNA to the luminal membrane of IC in distal tubules and CCD of rabbit kidneys was first demonstrated by LeHir *et al.* (1982). Using the gill epithelium of rainbow trout, our lab has also shown *in situ* binding of PNA to cells located in the base of lamellae, and in the interlamellar region of filament towards the trailing edge (Goss *et al.*, 2001). Cortisol treatment has been shown to increase the number of MR cells in the gill epithelium (review by Perry, 1998), and the increase in PNA staining in situ after 6 days cortisol treatment supports the hypothesis that the PNA was binding to MR cell sub-types. This was further supported by electron microscopy of the dispersed gill cells.

The objective of this research was to functionally characterize the roles of the MR cell sub-types. This necessitates a method to isolate relatively pure populations of these MR cell sub-types from a heterogeneous dispersed gill cell population. To accomplish this, we developed a magnetic bead separation system (MACS) to isolate PNA^+ cells from the MR rich population obtained by discontinuous density separation. Using this technique we were able to isolate a population of PNA^+ cells with a >95% purity (Galvez *et al.,* 2002). The PNA^- fraction was also found to be comprised primarily of MR cells, but some contamination of other cells, such as red blood cells, can be found in this fraction. A marker for the PNA^- fraction that could

be used in the magnetic bead separation system would be of great benefit, but is not available yet.

Differential staining of PNA to the MR cells in the gill epithelium suggests that these PNA sub-types could have distinct protein expression and thus, could also show distinct functional properties when presented with acid/base disturbances. Therefore, we used Western blotting to examine the differential expression of both Na^+/K^+- and H^+-ATPase in the PNA^+ and PNA^- cells during acid/base disturbance by respiratory acidosis and metabolic alkalosis. If a functional separation exists between these two PNA fractions, then we hypothesized that this might be realized by differential expression of Na^+/K^+-ATPase between the two cell types. During hypercapnia, we found that the Na^+/K^+-ATPase ratio of PNA^-:PNA^+ cells increased from 0.3 to 0.8. Whole animal experiments have shown that during hypercapnia there is an increase in H^+ excretion and Na^+ uptake (Goss *et al.,* 1992). Also, it has been demonstrated that Cl^- uptake and HCO_3^- secretion are decreased during hypercapnia (Hyde and Perry, 1989). This increase in the ratio of Na^+/K^+-ATPase expression could be indicative of an increase in the activity of the PNA^- cells, a decrease in activity of the PNA^+ cells, or both.

The current model of Na^+ transport in the gill suggests that the transport of Na^+ through an apical channel is rheogenically coupled to a H^+-ATPase transporter (Perry, 1997). Sullivan *et al.* (1995) have demonstrated expression of the 31-kDa subunit of the V-type H^+-ATPase increases during hypercapnia. Further, this research showed localization of the H^+-ATPase exclusively in the pavement cells of freshwater rainbow trout. Alternatively, Lin and colleagues (Lin *et al.,* 1994; Lin and

Randall, 1991) and Wilson *et. al.* (Wilson *et al.,* 2000a; Wilson *et al.,* 2000b) have demonstrated H^+-ATPase immunoreactivity in both the pavement cells and the MR "chloride cells". Using our PNA populations, we demonstrate H^+-ATPase in both fractions, but only the PNA^- cells show increased expression of the H^+-ATPase during acidosis. This increase in H^+-ATPase expression was not noticed during either NaCl or $NaHCO_3$ infusion, suggesting the site of H^+ excretion is likely the PNA^- cells. The pavement cells have been linked to H^+ excretion and Na^+ uptake in previous research (Goss *et al.,* 1998; Perry, 1997; Girard and Payan, 1980), agreeing with our findings. This data is intriguing, but a limitation of Western blotting is that we are unable to determine if the immunoreactivity is on the plasma membrane, or if it is in the intracellular compartment. An apical transport protein (*i.e.* apical H^+-ATPase) would require the immunoreactivity to be on the plasma membrane. Another potential problem is that we see peripheral staining of PNA on our PNA^+ cells, indicating that the cells have lost polarity due to cell dispersion techniques. Development of a viable cell culture system would enable the cells to regain polarity and proper cellular functioning.

The radioactive $^{22}Na^+$ influx data confirms that Na^+ uptake in the MR cells is most likely via an epithelial Na^+ channel (ENaC type) electrically coupled to an H^+-ATPase. We have also demonstrated that the phenamil-sensitive Na^+ uptake is located in the PNA^- MR cells. This location was based on the use of a specific ENaC blocker, phenamil. This blocking agent was effective in reducing the acid-stimulated Na^+ influx, but only in the PNA^- cell type. Also, with the addition of bafilomycin (an H^+-ATPase inhibitor) the acid-induced Na^+ influx was also reduced to control levels.

This data strongly supports the hypothesis that Na^+ uptake is via an ENaC type channel linked to an H^+-ATPase located in the PNA^- MR gill cells.

This data helps verify that the functional differences in the PNA^+ and PNA^- cells might be attributed to separate roles in acid/base and ion regulation, such as in the α- and β-IC in the mammalian kidney. We hypothesize that the PNA^- cells are analogous in function to the acid secreting α-cells of the mammalian CCD, and the PNA^+ cells are analogous to the base secreting β-cells. The apical anion exchanger from the fish gill has not yet been cloned. Future work in our lab is focused on identifying the molecular identity of the analogous apical anion exchanger from the fish gill.

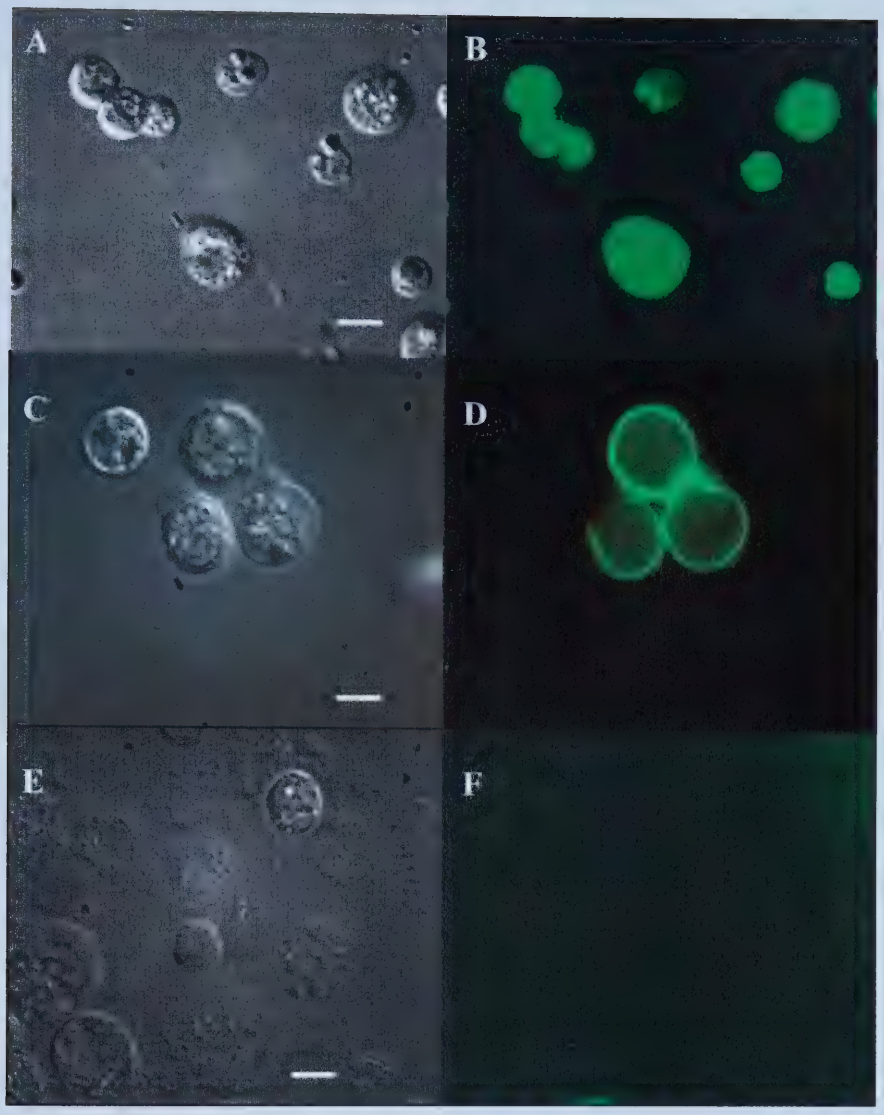

Figure 2.1

DIC and fluorescence images of MR cells. Frames A, C and E show DIC images of MR cells. Frame B shows the fluorescent image of frame A with Mitotracker Green-FM (100 nM, 20 min) staining of MR cells to demonstrate that these cells are mitochondria-rich. Frame D shows PNA-FITC (40 µg/mL) flourescent field of frame C demonstrating PNA^+ MR cells. Frame F depicts PNA-FITC fluorescence of PNA^- MR cells (with no detectable apical staining). Scale bars = 10 µm.

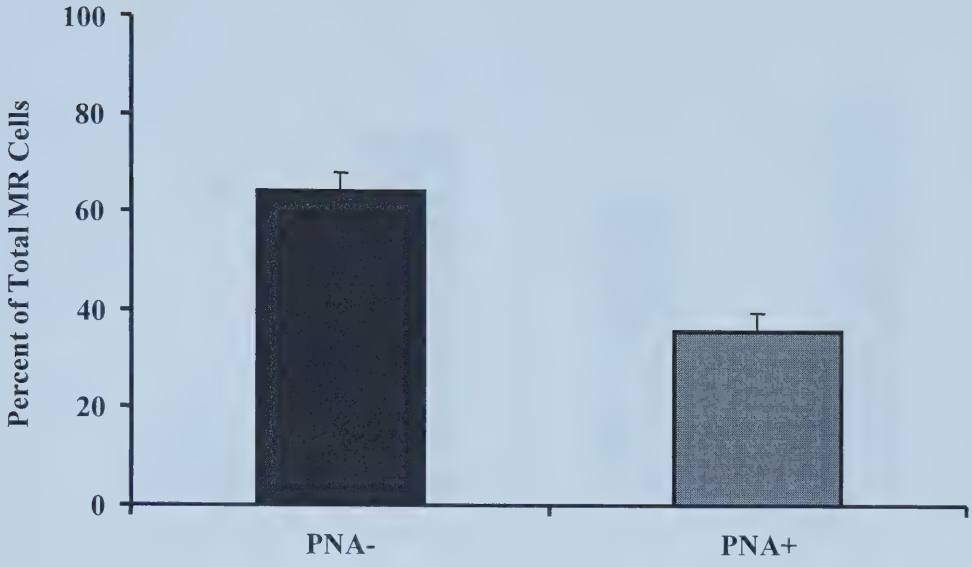

Figure 2.2

Relative abundance of PNA^- and PNA^+ cells in the total MR gill cell fraction. MR Cells were collected by discontinuous Percoll gradient and stained with PNA-FITC (40 μg/mL) for viewing with fluorescence microscopy to determine percentage of total cells that were PNA^+ and PNA^-.

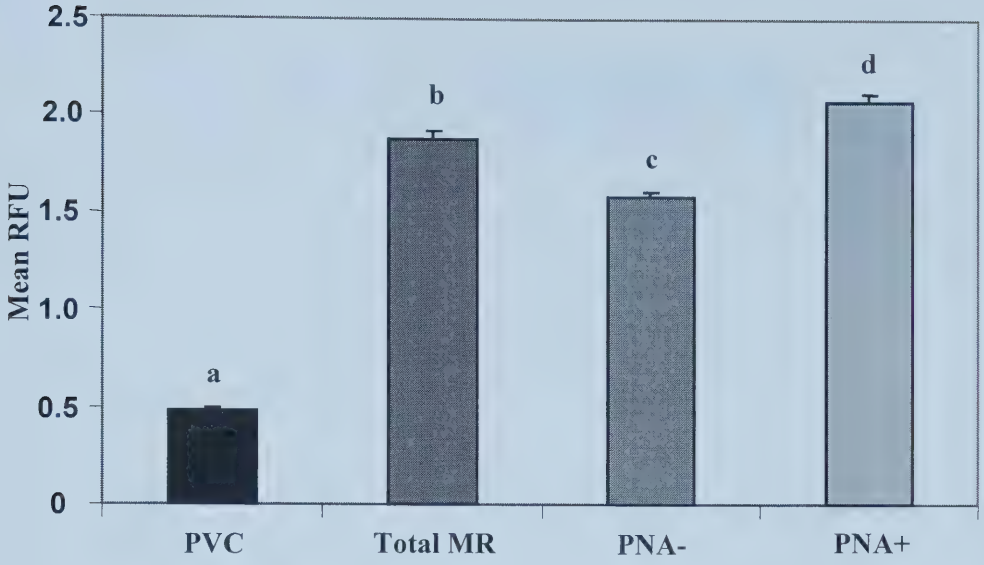

Figure 2.3

Mitotracker staining of gill cells from MR fraction and pavement cell (PVC - non MR) fraction. Cells from the total MR fraction were separated into PNA sub-types using MACS separation system. Cells incubated with Mitotracker Green – FM (100 nM, 20 min) and analyzed for relative fluorescence using a fluorescence plate reader. Values represent means ± SE (n = 4 fish). Significant differences ($P<0.05$) are denoted by different letters.

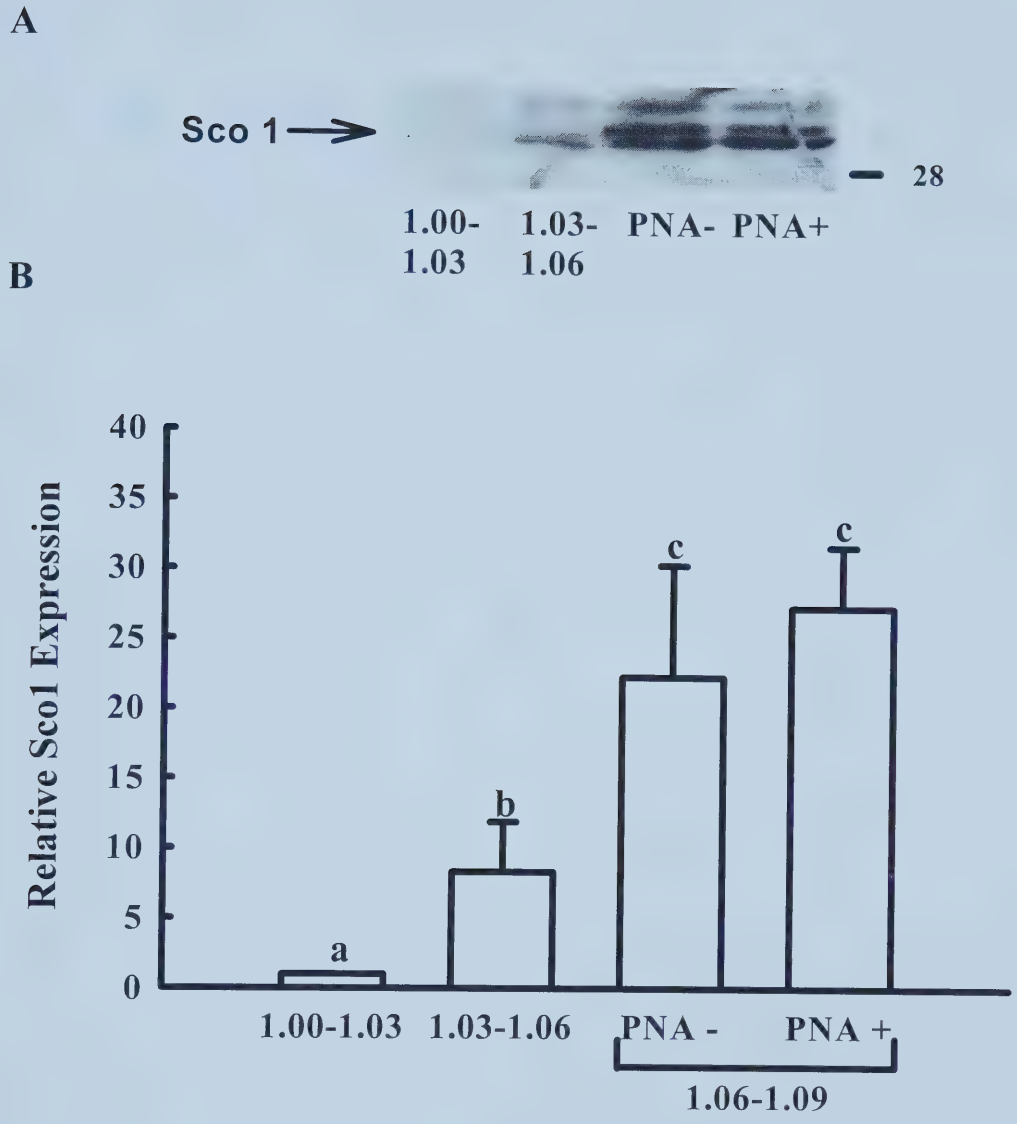

Figure 2.4

Relative expression of Sco1 in cells isolated from interfaces of discontinuous Percoll density gradients. Cells migrating to the 1.05–1.09 g/ml Percoll interface (total MR) were further separated into PNA+ and PNA- fractions using MACS. Cells (200,000 cells/lane) were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and probed using an antibody against an integral mitochondrial membrane protein from yeast (Sco1). *A*: representative blot. *B*: relative Sco1 levels in cell fractions were normalized to the expression levels of the PVC fraction. Values represent means ± SE ($n=4$ *fish*). Significant differences ($P<0.05$) between mean values are denoted by different letters.

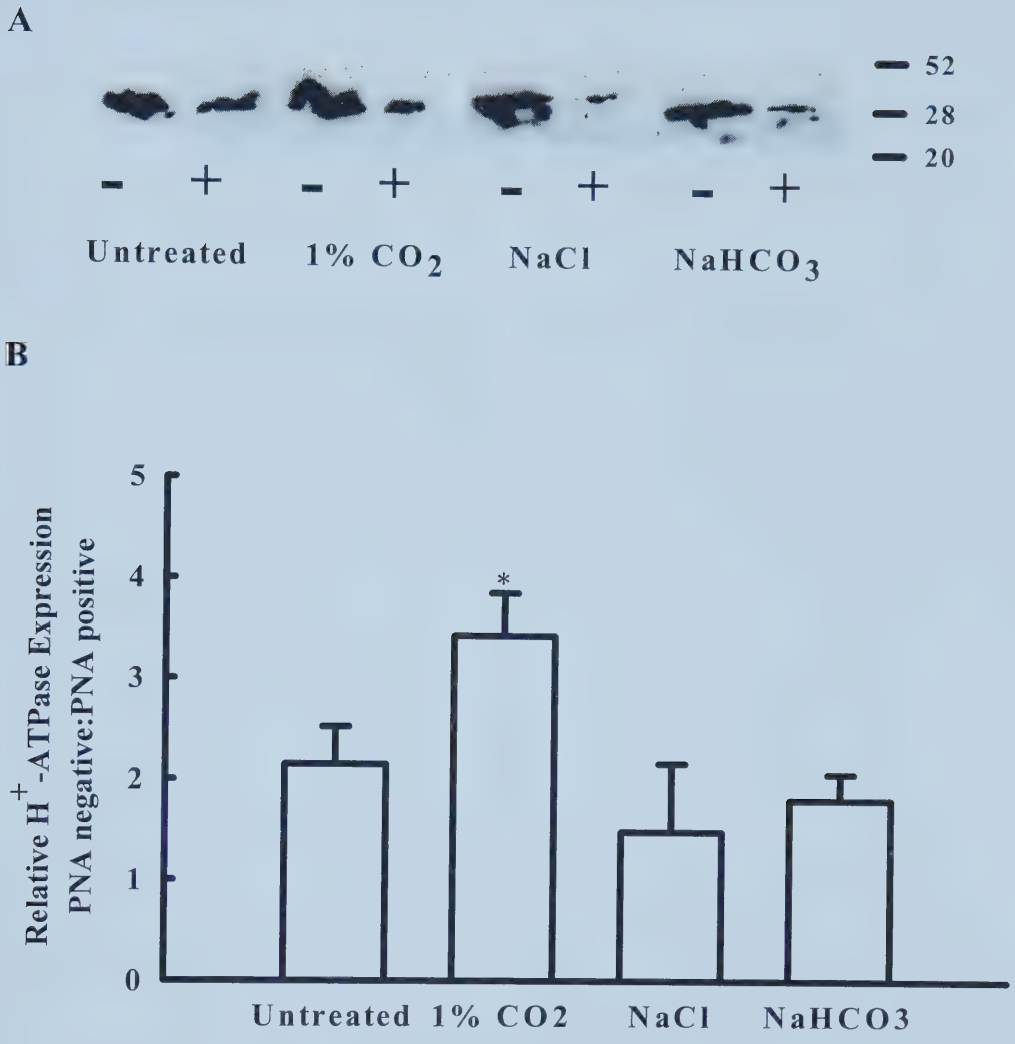

Figure 2.5

Dispersed gill cells were harvested from the MR gill cell fraction of fish exposed to 1% CO2 (hypercapnia), 140 mM NaHCO3 infusion, 140 mM NaCl infusion (infusion control), or untreated for 24 h. PNA- and PNA+ fractions were obtained using magnetic cell sorting. Western blots (5 μg protein/lane) of cell fractions separated on 10% SDS-polyacrylamide gels and probed for an antibody against a peptide in the 31-kDa subunit of bovine V-type H+-ATPase. *A*: representative blot showing molecular mass markers at 28 and 52 kDa. *B*: values are means ± SE (n = 4–6 fish) of the ratio of expression in PNA- to PNA+ fractions. Significant difference indicated by symbol.

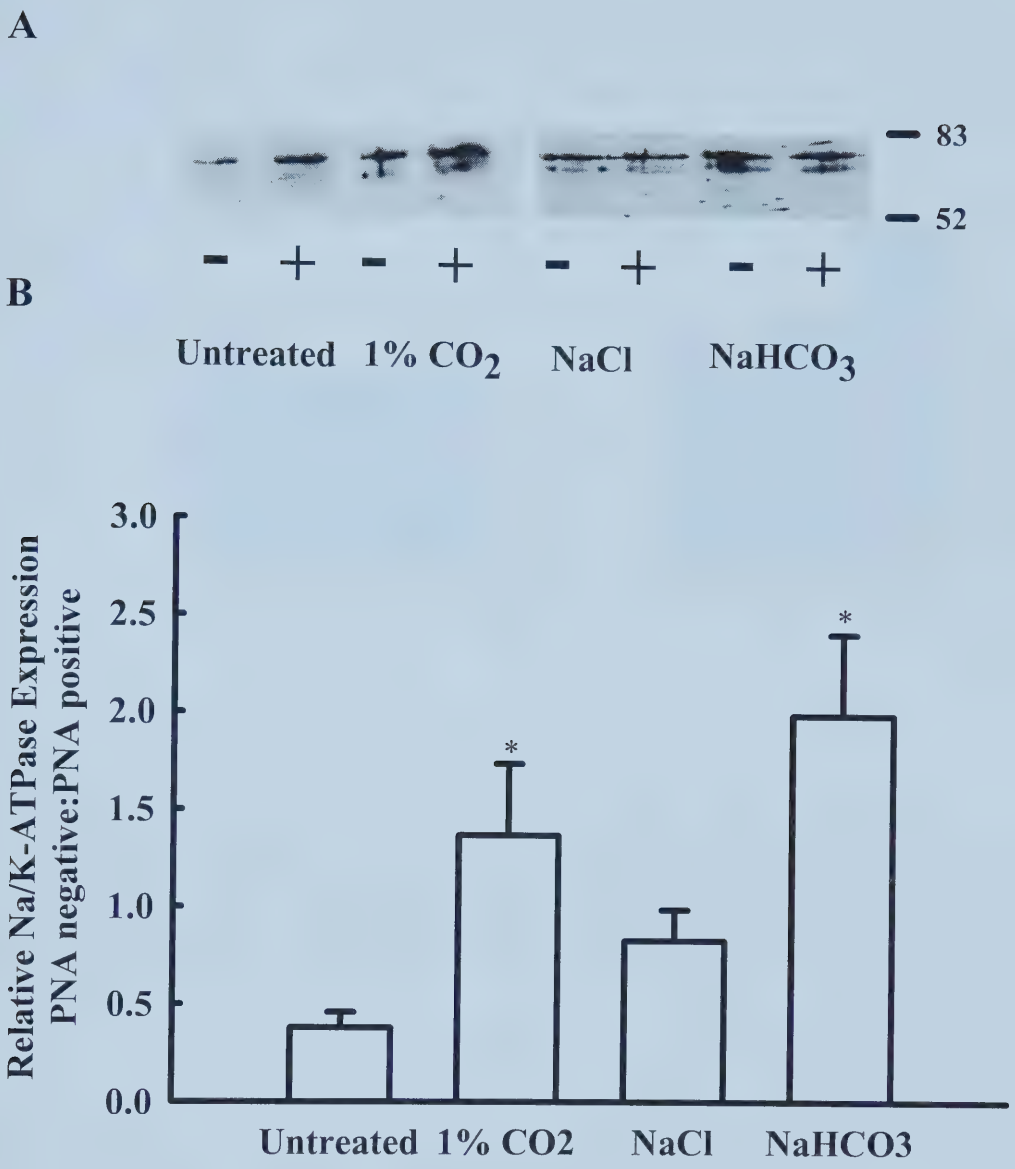

Figure 2.6

Dispersed gill cells were harvested from the 1.06–1.09 g/ml Percoll fractions of fish exposed to 1% CO2 (hypercapnia), 140 mM NaHCO3 infusion, 140 mM NaCl infusion (infusion control), or untreated for 24 h. PNA^- and PNA^+ fractions were obtained using magnetic cell sorting. Western blots (5 μg protein/lane) of cell fractions separated on 10% SDS-polyacrylamide gels and probed for an antibody against the Na^+/K^+-ATPase α-subunit. *A*: representative blot showing molecular mass markers at 52 and 83 kDa. *B*: values are means ± SE (n = 4–6) of the ratio of expression in PNA^- to PNA^+ fractions. Significance indicated by symbols.

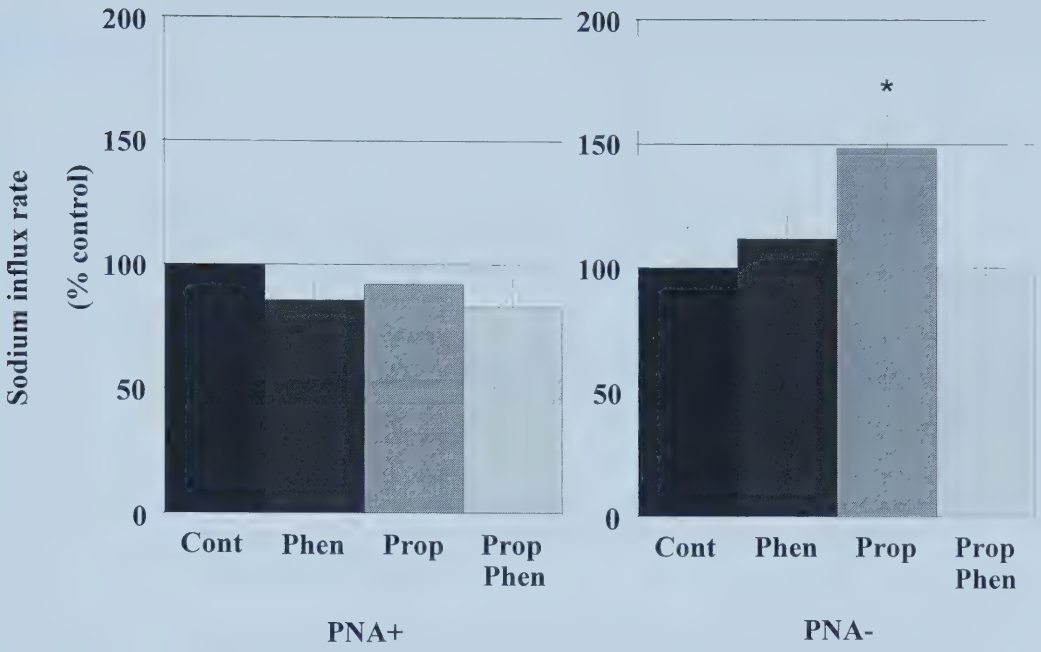

Figure 2.7

Radioactive $^{22}Na^{+}$ fluxes in PNA^{+} and PNA^{-} MR cells isolated using MACS isolation from the total 1.06 – 1.09 g/mL discontinuous Percoll interface. Na^{+} influx rates were determined in the absence of any additional inhibitors (control (cont)), 10 μM phenamil (phen), propionic acid alone (prop) or propionic acid and 10 μM phenamil (prop – phen). Graph represents means ± SE (n=4). Significance ($P<0.05$) designated by symbol.

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Chapter 3: Seawater Acclimation Causes Independent Alterations in Na^+/K^+- and H^+-ATPase Activity in Isolated Mitochondria-rich Cell Sub-types of the Rainbow Trout Gill.

Introduction

In freshwater fish, the current model for ion exchange links Cl^- uptake to HCO_3^- secretion via an anion exchanger. Meanwhile, Na^+ uptake is thought to occur via a Na^+ channel linked electrochemically to a coupled V-type H^+-ATPase on the apical membrane (Wilson *et al.,* 2002; Sullivan *et al.,* 1995; Lin and Randall, 1991). This model is based on the requirement to move both Na^+ and H^+ against their electrochemical gradients in low ionic strength media (Avella and Bornancin, 1989). However, in a higher ionic strength environment such as seawater, the Na^+ concentration gradient is favourable for linked electroneutral exchange. The mechanism by which the removal of H^+ is believed to occur is via an electroneutral sodium/proton exchange (NHE) system (Wilson *et al.,* 2000; Claiborne *et al.,* 1999) reducing the ATP requirement for acid-base regulation. NHE isoforms have been identified by molecular and immunological methods in the gills of a marine fish species (*Myoxocephalus octodecimspinosus*) and the euryhaline killifish (*Fundulus heteroclitus*)(Claiborne *et al.*,1999). Seawater acclimation results in many changes in gill function including increases the activity of Na^+/K^+-ATPase in MR gill cells

(Mancera and McCormick, 2000) and concomitant reductions in H^+-ATPase (Lin and Randall, 1993).

Recently, it has been proposed that there exist sub-types of the MR cells and the most recent work from our lab suggests that these sub-types perform different ionoregulatory functions (see Chapter2). Pisam *et al.* (1987) described two MR cell types (α and β) in the gills of freshwater teleost fish, but found that only one MR cell subtype existed in the gills of seawater teleost fish. More recently, Wong and Chan (1999) used flow cytometry to track the change in MR gill cell populations during seawater transfer using relative cell size, granularity and autofluorescence as the defining characteristics. They demonstrated that in freshwater Japanese eels, there are two distinct populations of cells that undergo a transition into morphologically separate seawater type cells. However, this study did not further assess function of these presumed MR gill cell populations. Nonetheless, these studies suggest existence of different MR cell sub-types during seawater acclimation.

The method we have developed for separation and isolation of functionally different MR cell sub-types based on differential binding to PNA can be used to assess changes in function and distribution of MR cells during a normal physiological function, seawater adaptation. The purpose of this study was two-fold. The first objective was to examine changes in MR cell sub-type populations (PNA^+ and PNA^-) during seawater acclimation to compare with the morphological data obtained by Pisam and colleagues (Pisam *et al.*, 1987) and Wong and Chan (1999). The second objective was to use our ability to isolate sub-populations of MR cells to

independently examine changes in cellular function in each of the MR cell sub-types during seawater acclimation.

Methods

Experimental animals

Freshwater rainbow trout (*Oncorhynchus mykiss*) of either sex were obtained from Alberta Trout Growers Ltd. and held exactly as in Chapter 2. Fish used in the seawater transfer experiments were removed from the holding facility and transferred to a recirculating system containing 10 parts per thousand (ppt)(~30% full-strength) seawater. The water temperature and photoperiod were unchanged from the acclimation conditions. Seawater was made by diluting Instant Ocean brand salts in dechlorinated Edmonton tap water. Fish were acclimated to 10 ppt for at least one week before transfer to a second system containing full-strength seawater (30 ppt) for at least an additional week before sampling. Salinity of seawater was checked weekly and adjusted, as needed. Experimental animals were randomly selected from holding tanks and killed by overdose (1 g/L MS-222, Syndel) followed by cephalic blow.

The Materials used, gill digestion protocol and separation of cells into fractions using the MACS system were exactly as outlined in Chapter 2.

Fluorescence microscopy

The percentage of MR cells binding PNA was determined by labeling cells with a biotin-conjugated PNA (40 μg/mL, 20 min), washing the cells in PBS, followed by a double labeling with streptavidin-Alexa fluor 594 (20 μg/mL, 15 min) and Mitotracker green-FM (40 μg/mL, 15 min). Cells were washed in PBS to remove unbound dyes and placed on glass slides for digital capture of either Mitotracker green-FM and/or Alexa fluor 594 images as performed in Chapter 2. Images were

binned at 2 x 2 to increase the sensitivity of fluorescence capture and final images were adjusted using Adobe Photoshop 6 for contrast and brightness only.

To determine the percentage of total MR cells made up of PNA^+ or PNA^- cells in the gills of freshwater and seawater acclimated trout, random view fields were selected on the slide and a DIC image captured for total cell counts. Serial images of each field were captured with fluorescence microscopy. Mitotracker green-FM fluorescence was used to indicate the MR cells in the field and Alexa fluor 594 fluorescence was used to determine the percent of MR cells that were PNA^+. At least 5 random fields were captured and used for each fish. Cell sizes were measured using the calibrated measuring tool located in Slidebook v.3.1.2 by Intelligent Imaging Innovations (Denver, Co., USA) with the DIC images captured from the seawater adaptation experiments.

ATPase assay

An ATPase assay based on a method developed by McCormick (McCormick, 1993) was adapted to determine both the ouabain (Na^+/K^+-ATPase inhibitor) and bafilomycin (V-type H^+-ATPase inhibitor) sensitive ATPase activities. Gill cells were counted using a haemocytometer and used as either total MR cells, or separated into PNA^- and PNA^+ fractions and stored in SEI buffer (in mM: 250 sucrose; 10 Na_2EDTA; 50 imidazole and adjusted to pH 7.3) at -80 °C until assays were performed. Cells were thawed and homogenized with the addition of 0.5% Na deoxycholic acid on ice and immediately centrifuged at 5000g for 30 seconds to remove insoluble material. Homogenate (10 μL) from each sample was added to 9

wells in a 96-well plate. This provided 3 treatments for each sample [Control, ouabain (500 μM) and ouabain (500 μM) + bafilomycin (50 nM)] with triplicate measurements of each treatment. Preliminary experiments demonstrated that H^+-ATPase activity in total MR cells was maximally inhibited between 10 and 50 nM concentrations of bafilomycin. Therefore, 50 nM was chosen as the appropriate dose for our assay. To each well was added 150 μL of assay mixture (in mM: 50 imidazole buffer; 2 PEP; 0.16 NADH; 0.5 ATP; in U/mL: 3.3 LDH; 3.6 PK) with appropriate drug treatment added and 50 μL of salt solution (in mM: 50 imidazole; 189 NaCl; 10.5 $MgCl_2$; 42 KCl). The microplate was read at a wavelength of 340 nm in a kinetic microplate reader (ThermoMAX) at 15 second intervals for 25 minutes. The average rate for each treatment was taken from the stable slope and calculated from a standard curve generated just prior to the assay. Na^+/K^+-ATPase activity was obtained by subtracting the ouabain-treated ATPase activity from control ATPase activity (see McCormick, 1993). We also modified the assay to assess H^+-ATPase activity by calculating the difference in ATPase activity between the ouabain and the oaubain + bafilomycin treated samples.

Statistical Analysis

All statistical measures were performed with SPSS version 10, using ANOVA followed by multiple comparison tests using Tukey's HSD. In all cases, the level of significance was set at $P<0.05$.

Results

Seawater acclimation is commonly associated with elevated levels of gill Na^+/K^+-ATPase activity (Tipsmark *et al.,* 2002; Mancera and McCormick, 2000). Using the total MR gill cell population (i.e. Percoll separated MR cells that have not been separated into PNA^+ and PNA^- sub-types), we found that acclimation to 10 ppt or 30 ppt resulted in a four- to five-fold increase in Na^+/K^+-ATPase activity on a per cell basis when compared to freshwater (Figure 3.1).

Similarly, reductions in bafilomycin sensitive H^+-ATPase activity occur in the isolated total MR cell fraction (Percoll separated MR cells not separated into PNA^+ and PNA^- sub-types). When the total MR gill cell fraction was examined, bafilomycin sensitive H^+-ATPase activity declined 79% from 0.32 ± 0.07 in freshwater fish to $0.067 + 0.02$ nmol ADP/10^{-6} cells/min in full-strength seawater acclimated fish (Figure 3.2). Figure 3.3(A-C) shows DIC (A) and fluorescence microscopic images (B,C) from the same field of view for total MR cells (Percoll density isolated) from freshwater rainbow trout. The fluorescence images permit the identification of cells as MR cells using Mitotracker (Fig 3.3 B) and further distinguishing those MR cells as either binding PNA or not (Fig 3.3 C). Greater than 95% of the cells in the fields of view (Fig 3.3 A) were found to be MR cells as demonstrated in Fig 3.3 B. The percent of PNA^+ cells were calculated from the number showing PNA fluorescence (Fig 3.3 C). Figure 3.3 D-F shows DIC (D) and fluorescence images (E,F) of the same field of view for a 30 ppt acclimated (seawater) trout. Clearly, the relative number of PNA^+ cells in the field of view has greatly increased from the freshwater situation (Fig. 3.3 F). Also noted during the image capture of gill cells from seawater

acclimated rainbow trout is the increase in size of the PNA^+ cells relative to the size of PNA^+ cells from freshwater rainbow trout (Figure 3.4).

These fluorescence images were used to quantify the relative numbers of the different MR cell sub-types during transition from freshwater to seawater. In freshwater, the total MR cell fraction is primarily made up of PNA^- cells. However, in either the 10 ppt (not shown) or the 30 ppt acclimated fish, almost all MR cells in the observed fields are PNA^+ (Figure 3.5). Quantification of these changes was performed by counting the total number of MR cells, and the number of PNA^+ MR cells in each corresponding field, to give the percentage of the MR cells that were PNA^+ (minimum 5 fields per fish). In freshwater, approximately 35% of the MR cells are PNA^+ while after either 10 or 30 ppt seawater acclimation, between 78% and 94% of the MR cells are PNA^+ (Figure 3.5). Of note, this figure represents only the relative distribution of the PNA^+ and PNA^- cells and cannot distinguish between an increase in one cell type *versus* a decrease in another. Unfortunately, absolute cell counts are not possible due to losses of total cells throughout the protocol due to suction and handling of the cells, a major assumption of our methodology is that there are no differential losses of one MR cell sub-type over another. We believe that the relative distribution of cells should remain the same within a single fraction in a single isolation. A potential complication was that the mobility of PNA^- cells in the Percoll gradients might have changed during acclimation of fish to seawater. However, MR cells were not detected in any of the other Percoll fractions (data not shown) indicating that seawater acclimated MR cells are in fact all migrating to the

same Percoll density fraction as found for freshwater MR cells and that the change in relative distribution of PNA^+:PNA^- cells is real.

The total MR cell fraction was separated into PNA^- and PNA^+ using the magnetic bead separation system and ouabain-sensitive (Na^+/K^+-ATPase) activity measured. The PNA^+ fraction showed a decrease in Na^+/K^+-ATPase activity, on a per cell basis, upon transfer of rainbow trout from freshwater to 100% seawater while the PNA^- fraction demonstrated the opposite response. PNA^- Na^+/K^+-ATPase activity (on a per cell basis) was found to significantly increase during acclimation to full strength seawater (Figure 3.6).

We separated the total MR gill cells into PNA^+ and PNA^- fractions and assessed bafilomycin-sensitive H^+-ATPase activity. Under freshwater conditions, H^+-ATPase activity in PNA^- cells was the same as in PNA^+ cells and was substantially (~90%) decreased after acclimation to either 10 or 30 ppt (Figure 3.7). Interestingly, we also found that H^+-ATPase in the PNA^+ cells of freshwater trout was also high and a similar trend occurred with a 73% reduction in activity occurred during seawater acclimation.

Discussion

Previous reports from our lab have established that there are at least two functionally distinct sub-populations of MR cells based on *in situ* electron microscope work (Goss *et al.,* 2001) and fluorescence microscopy in live dispersed gill cells (Galvez *et al.,* 2002). This previous work correlates with the earlier morphological studies of Pisam and colleagues (Pisam *et al.,* 1990; Pisam *et al.,* 1987) and Wong and Chan (1999) suggesting different sub-types of gill MR cells. The present data are the first to show evidence for changes in function and relative distribution of specific MR cell sub types during seawater acclimation. Pisam and colleagues, using transmission electron microscopy, demonstrated that there are lighter and darker staining MR cells in gills of freshwater fish which they termed α and β, respectively (Pisam *et al.,* 1987). Furthermore, during seawater acclimation, they found that β-cells disappeared leaving only α-cells as the MR cell in seawater fish (Pisam *et al.,* 1990). Similarly, Wong and Chan (1999) also found in Japanese eels, using flow cytometry, that there were two distinctly gated MR cell populations in freshwater while during transition to seawater, the size and internal complexity of the two MR cells populations increased. In freshwater, Wong and Chan demonstrated that two populations of MR cells existed; one type making up about 67% of the population and the other approx. 33%, values close to the distribution of PNA sub-types seen in our freshwater trout (35% PNA^+/ 65% PNA^-). Additionally, the major freshwater cell identified (F1 population) began disappearing by day 1 and was gone by day 3, a process which might appear in our study as a PNA^- cell-type reduction. The increased size of the remaining MR cells after seawater transition correlates with the

increased size noted for the PNA^+ cells isolated from seawater trout. This apparent increase in size concurs with the increase in size of MR cells during seawater acclimation as reported by other labs (Wong and Chan, 1999; Kultz *et al.,* 1992; Pisam *et al.,* 1987; Pisam, 1981). These previous studies lend support to our finding that there is a transition of cell types during seawater acclimation in fish. The current study is the first to link these changes with alterations in biochemical function in independently separated cell types.

Levels of Na^+/K^+-ATPase have been used extensively as an index of transport capacity in fish exposed to a variety of conditions including seawater transfer (Mancera and McCormick, 2000; Yoshikawa *et al.,* 1993). We have already demonstrated that the MR cell fraction possesses high levels of Na^+/K^+-ATPase activity compared to the other fractions in the Percoll density separation (Galvez *et al.,* 2002). Activity and abundance of Na^+/K^+-ATPase in total gill homogenates have been demonstrated to increase with seawater acclimation and have been used numerous times as "indicators" of readiness for migration or transition into seawater (see review McCormick, 1995). Our data are consistent with these previous findings that Na^+/K^+-ATPase activity is elevated in the total MR gill cell fraction during transition to seawater (Fig. 3.1). We demonstrate that Na^+/K^+-ATPase activity is approximately 5 fold higher in the gills of seawater-acclimated trout compared to that observed in the freshwater fish gill. This increase is in agreement with previously published figures (Wilson *et al.,* 2002; Kultz *et al.,* 1992; Epstein *et al.,* 1980). However, this paper is the first to separate and independently analyze this response in distinct MR cell sub-types. We found that the two cell sub-types respond differently

to seawater acclimation. While the Na^+/K^+-ATPase activity of PNA^+ cell types is significantly greater than that of the PNA^- cells under freshwater conditions, Na^+/K^+-ATPase activity in the PNA^- cells of seawater acclimated fish is increased while that of PNA^+ cells is decreased. These findings have implications for future interpretation of total Na^+/K^+-ATPase activity in gill cells where differential changes in MR cells distribution and function could be occurring.

The current model for ion exchange in the cells of freshwater fish links Na^+ uptake to a rheogenically coupled H^+-ATPase. This H^+-ATPase drives the uptake of Na^+ through an apical epithelial Na^+ channel (likely a phenamil senstive ENaC type Na^+ channel although direct molecular evidence is lacking) by creating a negative potential inside the cell (see review Perry, 1997). We have previously shown that there is an acid-inducible, phenamil-sensitive Na^+ uptake attributed to the PNA^- cells in freshwater trout gills (Reid *et al.,* 2003). Furthermore, Western blot analysis shows that the expression of H^+-ATPase is elevated in the PNA^- cells, relative to that of PNA^+ cells, and increased during hypercapnia (acidosis), but not during infusion of bicarbonate (Galvez *et al.,* 2002). Of note, however, was the relatively high expression of H^+-ATPase in the PNA^+ cells under freshwater control conditions.

In seawater, the requirement for H^+-ATPase driven Na^+ uptake is reduced or eliminated due to a favourable Na^+ gradient into the fish. It has been shown that Na^+ movement in a seawater situation is more likely through an energetically favourable NHE mechanism (Claiborne *et al.,* 1999). Reductions in H^+-ATPase immunoreactivity (Piermarini and Evans, 2001; Lin *et al.,* 1994), and biochemical activity (Wilson *et al.*, 2002; Lin and Randall, 1993) have been previously

demonstrated during seawater acclimation in fish. These results are comparable to those seen in this study (Figure 3.2), with total MR cell H^+-ATPase activity decreased during seawater acclimation to about one third of the activity of the freshwater fish.

An important contribution of the present paper is the analysis of independent changes in H^+-ATPase activity in separate MR cell populations. When the total MR gill cell fraction was separated into PNA sub-types, we found that seawater adaptation resulted in a notable decrease in H^+-ATPase activity in both the PNA^- and the PNA^+ cells. The decreases in H^+-ATPase activity found in PNA^- cells during seawater adaptation were predicted based on previous attribution of Na^+ uptake to this particular cell type (Reid *et al.,* 2003). We were surprised at the relatively high levels of H^+-ATPase activity found in the freshwater PNA^+ cells and the concomitant reductions that occurred during seawater acclimation. In freshwater, the PNA^- cell type is responsible for Na^+ uptake as demonstrated in the paper by Reid *et al.* (2003). Acid-stimulated, phenamil-sensitive Na^+ uptake could only be found in the PNA^- cell fraction and not in the PNA^+ fraction. Theoretically, Na^+ uptake from low ionic strength media requires active H^+ extrusion to the water via an apical H^+-ATPase to provide the driving gradient for Na^+ uptake. This mechanism for Na^+ uptake was first proposed by Avella and Bornancin (Avella and Bornancin, 1989) based on the frog skin model of Na^+ uptake (Harvey and Ehrenfeld, 1988; Harvey and Ehrenfeld, 1986; Ehrenfeld *et al.,* 1985). A similar mechanism has also been demonstrated for acid-base regulation in the mammalian kidney (Brown and Breton, 2000). In the kidney, two types of MR cells exist, termed α- and β- (acid and base secreting, respectively) IC cells. These cells have similar morphological and physiological characteristics to

the MR cells in the fish gill. The results of this and previous experiments are consistent with this model and allow us to suggest that the PNA^- cell be termed the α-MR cell in the fish.

Of note, our terminology is inconsistent (opposite) with that of the α- and β-MR cells coined by Pisam and colleagues (Pisam *et al.,* 1987). Our terminology follows functionally from the α- (acid-secreting) and β- (base-secreting) IC cells in the mammalian kidney and is based primarily on that fact that the PNA^- cells types in both tissues are responsible for Na^+ uptake and acid-excretion while the PNA^+ cells in the kidney have been demonstrated to be involved in base-secretion (apical Cl^-/HCO_3^- exchange). Pisam and colleagues (1987) based their observations on morphological (staining) characteristics which, unfortunately, do not match the functional equivalents in the mammalian kidney.

During seawater acclimation, external Na^+ rises to a level that favours inward directed Na^+ movement without the need for an energetically expensive apical H^+-ATPase. Our results, showing a reduction in PNA^- (α-MR cell) H^+-ATPase during seawater acclimation are consistent with this model. Other authors (Piermarini and Evans, 2001; Lin *et al.,* 1994) have also documented reduction in H^+-ATPase immunoreactivity during seawater acclimation and also the appearance of immunoreactivity of the NHE-2 isoform of the Na^+/H^+ exchanger family in seawater adapted fish (Wilson *et al.,* 2002).

The present findings of elevated H^+-ATPase activity in PNA^+ cells was unexpected as were the changes that occurred during seawater acclimation. This suggests that H^+-ATPase may play a significant role in Cl^- uptake and acid-base

regulation in freshwater fish. Cl^- uptake from very dilute freshwater does not have a favourable driving electrochemical gradient from the water to the fish. Similarly, a strict gradient for HCO_3^- from the blood to the water is not sufficient to provide a driving gradient for HCO_3^- excretion under normal conditions. We propose that Cl^- uptake is linked to the activity of H^+-ATPase in PNA^+ MR cells from the freshwater trout gill, although the mechanism is as yet undetermined. Immunocytochemical localization of Pendrin-like (AE in β-IC cells) immunoreactivity has been demonstrated for euryhaline stingrays (Piermarini *et al.,* 2002) but has not yet been demonstrated for salmonids. Our future research focus will take advantage of our ability to isolated MR cells into separate populations and to use this technique to functionally characterize these individual cell populations and their adaptations during environmental changes. The power of this approach is demonstrated in the above results, whereby independent changes in biochemical activity in separate cell types are unmasked by the ability to separate the MR cells

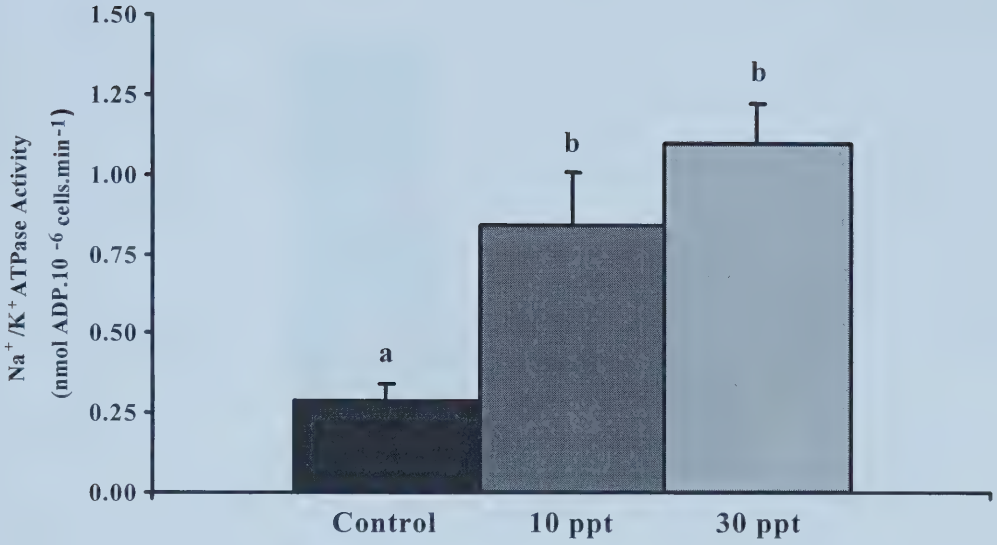

Figure 3.1

Ouabain sensitive Na^+/K^+-ATPase activity of control and seawater adapted fish in cells collected from the total MR 1.05-1.09 g/mL Percoll interface. Both partial strength seawater (10 ppt) and full strength seawater (30 ppt) adapted fish used for testing. Significant differences indicated by letters. Values represent means ± SE. (n = 5 fish).

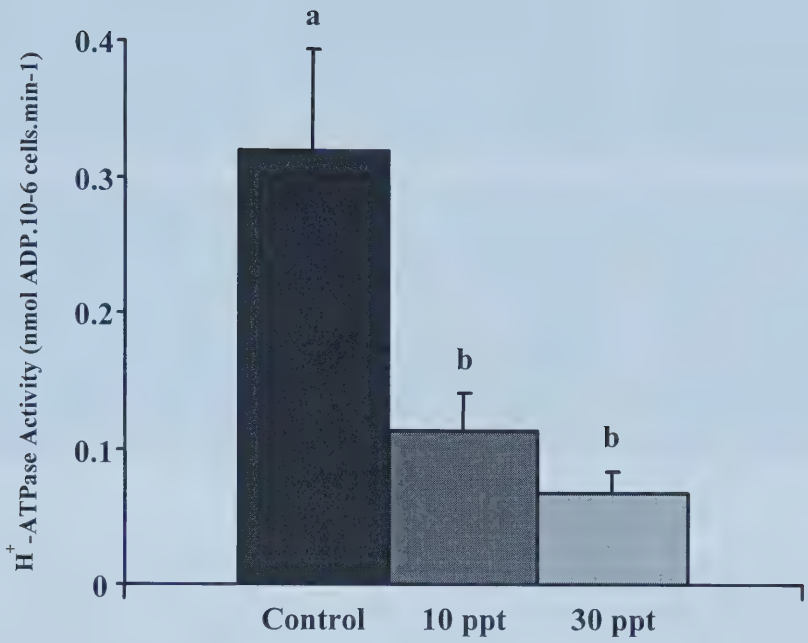

Figure 3.2

Bafilomycin (50 nM) sensitive H^+-ATPase activity in the total MR cell fraction collected from the 1.05-1.09 g/mL Percoll interface. Freshwater control fish compared to both 10 ppt and 30 ppt seawater adapted fish. Significant differences indicated by letters. Values represent means ± SE. (n = 4-6 fish).

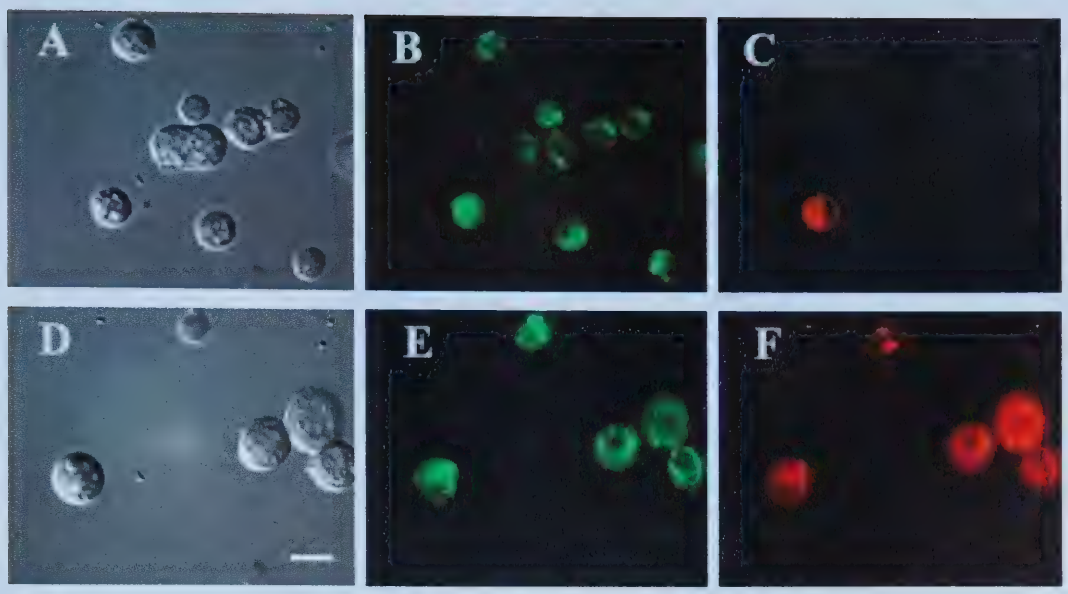

Figure 3.3

Differential Interference Contrast (DIC) and fluorescence images of dispersed gill cells from freshwater rainbow trout (A,B,C) and seawater adapted (30ppt) trout (D,E,F). Cells were obtained from the mitochondria rich (MR) 1.05-1.09 g/mL Percoll layer and stained with Mitotracker Green-FM (100 nM, 20 minutes) (B and E) or labeled with PNA-Alexa fluor 594 (40 µg/mL) (C and F) for identification of MR and PNA^+ cell types, respectively. All pictures in rows are of same field of view (*e.g.* A - C). Scale bar = 10 µm.

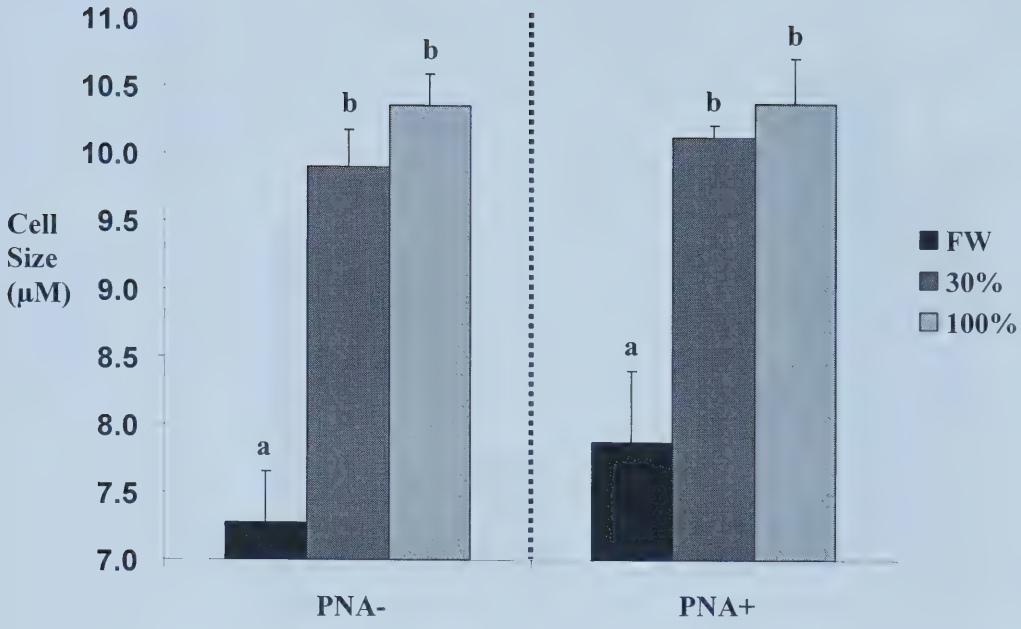

Figure 3.4

Measured diameters of cells in freshwater and seawater adapted trout. Cell sizes measured by calibrated measuring tool in viewing software from DIC images taken for seawater adaptation experiments. Values represent means ± SE (n = 30 – 40 cells for each population).

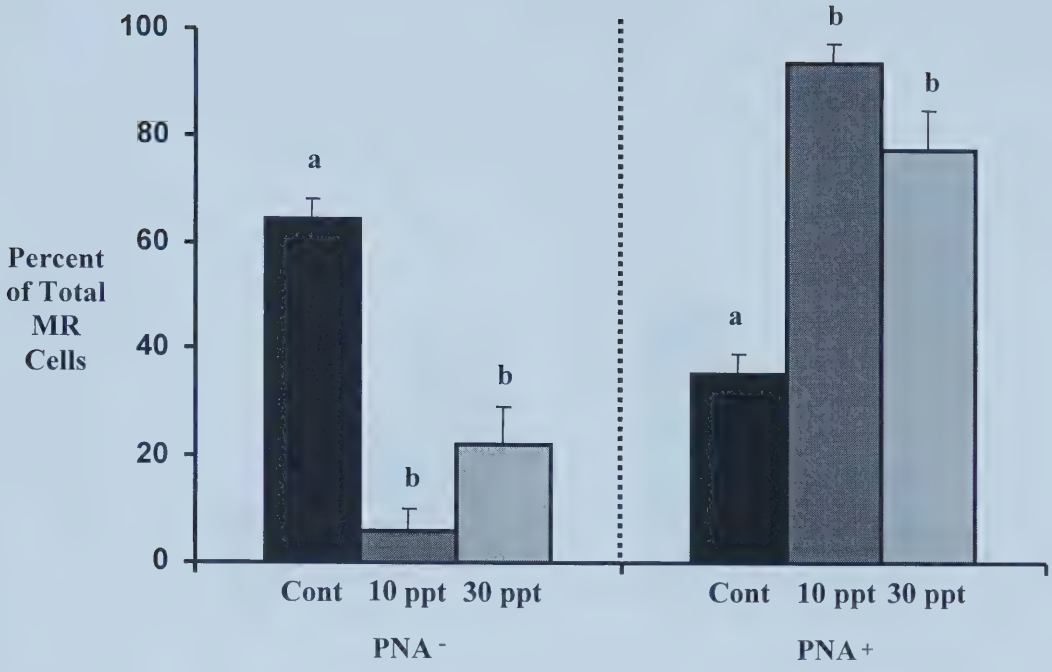

Figure 3.5

Relative percentage of PNA^- and PNA^+ cell types in populations of gill cells isolated from the MR 1.05-1.09 g/mL Percoll interface. Control fish (freshwater) are compared to seawater adapted fish at partial strength (10 ppt) and full strength seawater (30 ppt). PNA^+ and PNA^- cells counted under 100x objective of fluorescence microscope with a minimum of 5 fields of view counted for each freshwater and seawater fish. Values represent means ± SE. (n = 6 fish for each treatment group).

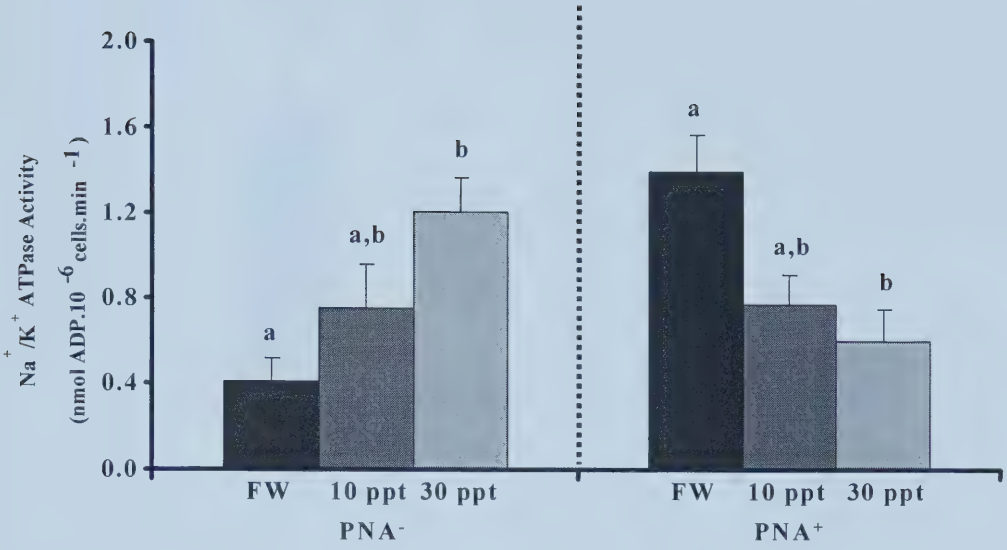

Figure 3.6

Ouabain sensitive Na^+/K^+-ATPase activity of control and seawater adapted fish in the PNA^- and PNA^+ cell fractions. Both partial strength seawater (10 ppt) and full strength seawater (30 ppt) adapted fish used for testing. Values represent means ± SE. (n = 5-6 fish).

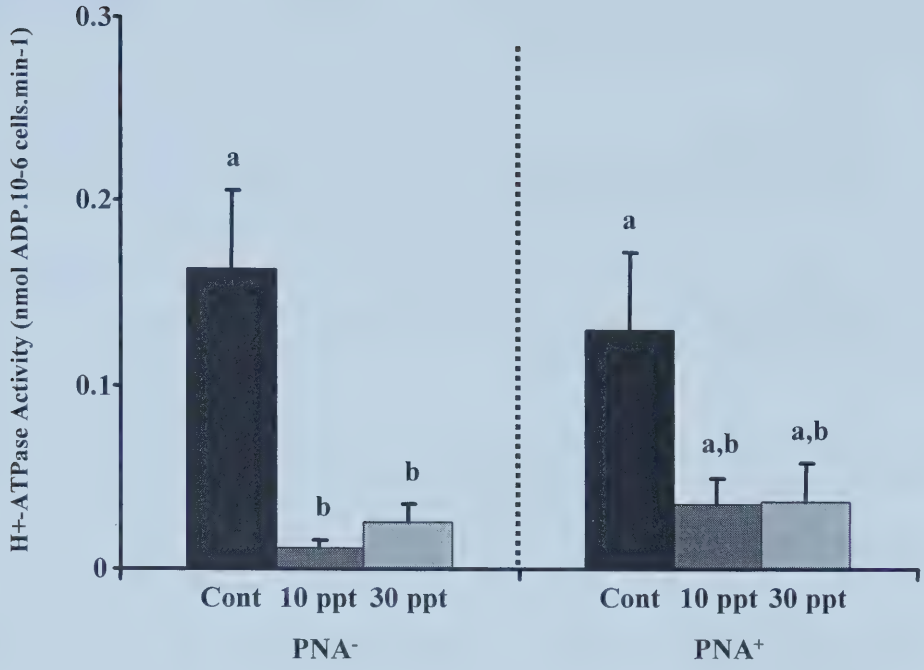

Figure 3.7

Bafilomycin (50 nM) sensitive H^+-ATPase activity in the PNA^+ and PNA^- gill cell fractions. Freshwater control fish compared to both 10 ppt and 30 ppt seawater adapted fish. Values represent means ± SE. (n = 3-5 fish).

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Chapter 4: Effects of Silver on Phenamil-sensitive Na^+ Uptake.

Introduction:

Silver, as silver nitrate ($AgNO_3$), has a 96 h half-maximal lethal concentration (LC_{50}) of between 5 and 70 µg total Ag/L, making it one of the most potent toxic metals to freshwater fish (Hogstrand and Wood, 1998). The lethality of silver is directly attributed to the amount of free Ag^+ present in the water, where water chemistry (ions present in the water) can significantly influence the amount of the free Ag^+ present. For example, anions such as Cl^- can readily bind up the Ag^+, rendering it non-toxic (Hogstrand and Wood, 1998). Free Ag^+ is known to be transported through the gills and exerts its toxic effect by interfering with Na^+ and Cl^- uptake mechanisms located on the gill (Morgan *et al.,* 1997). When Na^+ and Cl^- transport at the gill is significantly impaired, ion concentrations in the blood fall resulting in osmotic water shifts from the plasma, internal haemoconcentration and eventual cardiovascular collapse (Hogstrand and Wood, 1998; Wood *et al.,* 1996). One of the primary mechanisms of toxicity of Ag^+ is now thought to be through inhibition of the enzyme Na^+/K^+-ATPase (Morgan *et al.,* 1997). Once Ag^+ has entered the gill cell, it binds to the transport enzyme Na^+/K^+-ATPase, causing a conformational change within the molecule and the enzyme to malfunction (Morgan *et al.*, 1997; Karnaky *et al.,* 1976).

As mentioned previously in the Introduction, and demonstrated in Chapter 2, Na^+ uptake is linked rheogenically to a H^+-ATPase, whereby the action of the H^+-ATPase creates a favourable local electrochemical gradient for Na^+ entry through a phenamil-sensitive Na^+ channel (Perry, 1997; Lin and Randall, 1991; Avella and Bornancin, 1989). However, the molecular identification of the apical Na^+ channel remains to be determined (Reid *et al.,* 2003; Perry, 1997). Other toxic metals have also been reported to enter the fish gill cells through apical transport pathways such as Ca^{2+} channels. Divalent metals such as cadmium, zinc and cobalt have been demonstrated to enter the fish through apical Ca^{2+} channels, and the location of this transport is also postulated to be in the MR cells (Perry, 1997). The entry of the aforementioned metals can be competitively blocked by increasing the external Ca^{2+} concentration, giving strong evidence for entry of these metals through the apical Ca^{2+} channel (Marshall and Bryson, 1998; Comhaire *et al.,* 1994). Additionally, Ag^+ is known to inhibit whole body Na^+ uptake (Bury and Wood, 1999; Janes and Playle, 1995), although the exact mechanism of inhibition could not be established in these experiments, it was postulated to be via direct inhibition of the ENaC type Na^+ channel suggested to be on the gill epithelium.

Given this information, I hypothesized that the presence of Ag^+ would compete with the acid-inducible phenamil-sensitive Na^+ uptake demonstrated in Chapter 2 to be present only on the PNA^- MR cells. The Ag^+ ion has a much larger hydrated ionic radius than that of Na^+ and for this reason, it is thought to be transported at a much slower rate through the Na^+ channel, resulting in an effective blockade of the channel. I hypothesized the presence of Ag^+ would impair Na^+

transport though the PNA^- MR gill cells by acting as a competitive inhibitor to Na^+ uptake. Secondly, we want to determine if the Ag^+ that entered the cell acted to reduce ATPase activity in the cell as suggested by Morgan *et al.* (1997).

Methods:

Experimental animals

Adult rainbow trout (~200 g) (*Oncorhynchus mykiss*) were maintained exactly as described in Chapter 2.

Materials

The magnetic cell separation system and anti-FITC microbeads used for MACS separation were from Miltynei Biotech (Auburn, CA). Scintillation cocktail was obtained from ACS (Amersham, Baie d'Urfe, QC, Canada). Radioactive stock $^{22}Na^+$ was ordered through NEN (Boston, MA, USA). All other reagents used in the protocol were obtained from Sigma Chemicals, St. Louis, MO.

Na^+ influx experiments

Na^+ influx experiments were conducted as per methods in Chapter 2, with only minor exceptions. A confounding problem was the fact that Ag^+ reacts rapidly with Cl^- to form AgCl precipitates, rendering the Ag^+ non-toxic. To mimic a freshwater (low $[Cl^-]$) situation and to eliminate the potential for interaction of the Ag^+ with the Cl^-, the flux saline was made using a Cl^- free recipe (in mM: 157.5 NMG-nitrate, 5 K-gluconate, 1.5 Ca-gluconate, 1 Mg-gluconate, 5 HEPES (Na-free), 1 Na-gluconate; pH 7.8) so that there was no Cl^- available to bind with the Ag^+ in solution. Similarly, we used a lower Na^+ concentration (1 mM rather than 15 mM) in the flux solution to more closely mimic the freshwater $[Na^+]$. Cells were pre-incubated with Ag^+ ($AgNO_3$ diluted 1/10 upon addition to cells to give nominal final

concentrations of 10 μM, 1μM and 100 nM Ag^+) for 1 minute prior to addition of $^{22}Na^+$ and/or butyric acid in flux solution (final concentration of 20 mM). Cells were incubated in the flux solution for exactly 1 minute before addition to filters, washing of the cells (30 mL 154 mM NaCl wash solution) and analysis of $^{22}Na^+$ activity by scintillation counting.

Na^+/K^+- and H^+-ATPase measurements

Measurement of total ATPase was performed as previously outlined in Chapter 3 with minor revisions. The analysis of total ATPase activity and ouabain sensitive ATPase activity was exactly the same as outlined in Chapter 3. However, there were no inhibitory agents in the flux saline when tested with the $AgNO_3$ incubated cells. In essence, we used the $AgNO_3$ as a blocking agent, instead of ouabain, to examine the affect on total ATPase activity of the Ag alone.

Statistical Analysis

One way analysis of variance (ANOVA) and the post-hoc Bonferroni's Multiple Comparison test were performed using GraphPad Prism (v.3.0) to test for significant differences between control and experimental groups. $P<0.05$ was used as the fiducial limit of significance.

Results:

Na^+ influx experiments

Isotopic fluxes were used to measure $^{22}Na^+$ uptake by the gill cells. When $^{22}Na^+$ uptake analysis was performed using the cells from the 1.03 – 1.06 g/mL Percoll fraction (primarily pavement cells), there was no detectable significant difference in Na^+ uptake found, with all cells displaying a background $^{22}Na^+$ uptake of approximately 2 nmol/10^6 cells/min (Figure 4.1).

In the total MR gill cell fraction the control value of Na^+ uptake was 1.93 +/- 0.10 nmol Na^+/million cells/minute. Intracellular acidification, caused by the addition of 20 mM butyric acid for 1 minute, increased the Na^+ influx significantly to 5.33 +/- 0.43 nmol Na^+/million cells/minute (Figure 4.2). The addition of the H^+-ATPase blocker bafilomycin (10 nM; 1 minute pre-incubation) or the ENaC blocker phenamil (10 μM, 1 minute pre-incubation) both reduced the acid induced Na^+ influx measured to control values; 1.47 +/- 0.25 and 2.42 +/- 0.19 nmol Na^+/million cells/minute, respectively. Given that Ag^+ has been hypothesized to enter cells through the ENaC, but with a much slower permeation co-efficient, we examined the ability of Ag^+ to inhibit Na^+ uptake through the ENaC with increasing external concentrations from 100 nM to 10 μM $AgNO_3$. Incubation of 100 nM $AgNO_3$ resulted in a partial inhibition of $^{22}Na^+$ uptake, with a value of 3.80 +/- 0.42 nmol Na^+/million cells/minute. However, this was still significantly elevated above non-stimulated controls. Both 1 μM and 10 μM $AgNO_3$ caused a reduction of Na^+ uptake to values not significantly different from controls, measured at 2.86 +/- 0.18 and 2.82 +/- 1.2 nmol Na^+/million cells/minute, respectively.

In the next set of experiments, total MR cells obtained from the 1.06-1.09 g/mL Percoll fraction were then separated into PNA^- and PNA^+ fractions using the MACS system as outlined in Chapter 2. Only the PNA^- fraction was used in the Na^+ uptake experiments, as we have previously shown that this is the fraction responsible for phenamil-sensitive, acid-inducible $^{22}Na^+$ uptake. The control value for Na^+ uptake in the PNA^- cells was 1.87 +/- 0.07 nmol Na^+/million cells/minute (Figure 4.3). Internal acidification with 20 mM butyric acid caused the Na^+ to significantly increase to 6.00 +/- 0.25 nmol Na^+/million cells/minute. Both bafilomycin and phenamil incubations reduced the Na^+ uptake to control levels of 1.45 +/- 0.14 and 2.36 +/- 0.12 nmol Na^+/million cells/minute, respectively. Incubation with 100 nM AgNO3 reduced Na^+ uptake to 3.32 +/- 0.10 nmol Na^+/million cells/minute, a value intermediate between the stimulated value and the control unstimulated value. The addition of 1 μM and 10 μM both reduced $^{22}Na^+$ uptake to 2.68 +/- 0.27 and 2.67 +/- 0.25 nmol Na^+/million cells/minute, respectively, values not significantly different from the control unstimulated value.

ATPase measurements

The main enzyme reported to be interrupted by Ag^+ is the Na^+/K^+-ATPase. An ATPase assay was performed to see if the addition of Ag^+ to the intact MR gill cells had any effect on total ATPase function. We compared the Ag^+ incubated ATPase activity to the ouabain-sensitive (Na^+/K^+-ATPase) activity from control cells and examined if it was similar to the ouabain sensitive portion of the total ATPase activity of the cell. If these values were similar, then we could infer that the Ag^+ was

likely impairing Na^+/K^+-ATPase activity in the cell to the same extent as ouabain. When control cells were analyzed, all gill cell fractions showed approximately the same level of ouabain sensitive ATPase activity. With the addition of 10 μM $AgNO_3$ (1 min), the total MR cell fraction total ATPase activity showed an increased inhibition from 22.3% to 41.9% of the total ATPase activity (Figure 4.4). The addition of butyric acid did not significantly increase this inhibition from Ag^+ alone (42.5%).

The total MR cell fraction was separated using the MACS system and the PNA fractions were analyzed for inhibition of total ATPase activity. The PNA^+ cells showed no increase in inhibition of total ATPase activity with the addition of 10μM $AgNO_3$ and 10μM $AgNO_3$ plus acid (Figure 4.4). However, when the PNA^- cells were analyzed, there was a significant increase in inhibition of total ATPase activity from 24.0% to 38.4% and 34.4% with the addition of $AgNO_3$ and $AgNO_3$ plus acid, respectively (Figure 4.4). Again, the inhibition was not significantly increased above Ag^+ alone by the addition of butyric acid to the PNA^- cells.

Discussion:

In the present study we have shown that with the addition of either 1μM or 10 μM $AgNO_3$ the acid-induced Na^+ uptake in the PNA^- MR cells can be blocked. The protocol used in the current study was revised to give us a lower background, a better estimate of activity and a higher relative increase in acid-inducible $^{22}Na^+$ transport than our previous study (Reid *et al.,* 2003) by keeping external [Na^+] at 1mM, instead of 15 mM used previously. The present data is strong evidence supporting the hypothesis that Ag^+ uptake is via the H^+-ATPase linked Na^+ channel mechanism of believed to occur in the fish gill (for review see Perry, 1997). We have also shown that the addition, and entry, of Ag^+ into the MR cells causes a greater inhibition of total ATPase activity than ouabain alone.

Phenamil, which has been shown to be a potent inhibitor of the Na^+ channel (Kleyman and Cragoe, 1988; Garvin *et al.,* 1985), strongly inhibits the acid-induced $^{22}Na^+$ influx back to control values in PNA^- MR cells. This is evidence for the mechanism of Na^+ entry in the gill to be through an ENaC style Na^+ channel located on the PNA^- MR cells and recapitulates our earlier findings (Reid *et al.,* 2003) that Na^+ entry is via a phenamil-sensitive pathway. Also, addition of bafilomycin A_1, a potent H^+-ATPase inhibitor (Drose and Altendorf, 1997), also inhibited the acid-induced $^{22}Na^+$ influx. Earlier research has also demonstrated that H^+-ATPase activity can be significantly inhibited in rainbow trout gill homogenates (Lin and Randall, 1993) by bafilomycin, but this was not localized to specific gill cell types. Further evidence has shown the proton coupled Na^+ channel is also present in the gills of brown trout (Nelson *et al.,* 1997). Our lab has also previously demonstrated the

inhibitory effects of phenamil and bafilomycin on acid-induced $^{22}Na^+$ uptake in PNA$^-$ cells (Reid *et al.*, 2003), and the results from this study strongly support the current gill model in which Na^+ uptake is linked to a H^+-ATPase.

The route of entry of Ag^+ is thought to be into fish via the Na^+ uptake mechanism located on the gill surface. Evidence supporting a common mechanism includes the fact that phenamil and bafilomycin and Ag^+ all act by inhibition of Na^+ uptake in whole fish (Bianchini *et al.*, 2002; Bury and Wood, 1999; Avella and Bornancin, 1989). The data in this present Chapter lends support to the current model whereby Ag^+ uptake is through the apical Na^+ channel in the gills. Our present study was designed to look at the acid-induced influx of Na^+, and whether or not Ag^+ was capable of blocking the uptake through the Na^+ channel. With the addition of only 100 nM $AgNO_3$, there was a reduction seen in the overall increase of Na^+ uptake in both the total MR cells and the PNA$^-$. With the addition of 1 and 10 μM $AgNO_3$ we were able to completely reduce the Na^+ uptake in the total MR cells and the PNA$^-$ cells to control values. This data correlates well with previous studies that were able to show that increasing ambient Na^+ concentrations reduced Ag^+ influx in the gills of rainbow trout (Bury and Wood, 1999; Janes and Playle, 1995). Also, our effective concentrations of Ag^+ used in this study relates very closely to the range of the 96 h LC_{50} for toxicity of Ag^+ in freshwater fish (Hogstrand and Wood, 1998).

Once Ag^+ enters the cell, the toxic action is believed to be the binding of free Ag^+ to the Na^+/K^+-ATPase, which causes a conformational change and the inhibition of the enzyme (Morgan *et al.*, 1997; Karnaky *et al.*, 1976). On the basolateral membrane of the fish gill MR cell, there is an abundance of Na^+/K^+-ATPase (Perry,

1997). The Na^+/K^+-ATPase allows for a quick extrusion of Na^+ from the inside of the cell to the blood, maintaining a stable intracellular Na^+ concentration (Perry (1997)). If Ag^+ enters a cell and binds to the Na^+/K^+-ATPase, causing a reduction in Na^+ transport capacity, then the fish could suffer from internal fluid-volume fluctuation and this could eventually cause cardiovascular collapse (Hogstrand and Wood, 1998; Morgan *et al.,* 1997; Wood *et al.,* 1996). When we examined the affect of Ag^+ on total ATPase activity we were surprised to see that the entry of Ag^+ into the MR cells causes a greater increase in total percent ATPase inhibition than ouabain alone. Ouabain is a specific blocker of Na^+/K^+-ATPase (Silva *et al.,* 1977) and when it is incorporated into the ATPase assay solution, should give an indication of the total ATPase activity that is ouabain sensitive by subtracting the control value from the ouabain incorporated value. In our control cells, this value for ouabain sensitive ATPase activity was approximately the same for all the cell fractions. However, when we incubated the cells with 10 μM $AgNO_3$, the inhibition of total ATPase activity was far greater than that of the ouabain specific activity in both the total MR cells and the PNA^- cells. This would indicate that the entrance of Ag^+ into these cells is causing more enzyme malfunction than just Na^+/K^+-ATPase. This might indicate that Ag^+ is binding non-specifically to proteins other than just the Na^+/K^+-ATPase (such as H^+-ATPase, Ca^{2+}-ATPase, etc.), adding to the effective toxicity of Ag^+ upon entrance into the gill cells. Also, if the uptake pathway for Ag^+ is through a Na^+ channel, then the higher values in the PNA^- cells are evidence that they are in fact the cells that contain the Na^+ channels. The high value for the total MR cell fraction could be explained by the fact that in a freshwater situation, the total MR cells are

~70% PNA^- cells and they could be masking the affect of the PNA^+ cells in this fraction. This study presents evidence that Ag^+ uptake is via an apical Na^+ channel located in the PNA^- cells, and once Ag^+ enters the MR gill cells its toxic affect on total ATPase activity shows that ouabain sensitive Na^+/K^+-ATPase may not be the only protein affected by the non-specific binding of Ag^+. Future research should focus on trying to elucidate the possible binding sites available to intracellular free Ag^+.

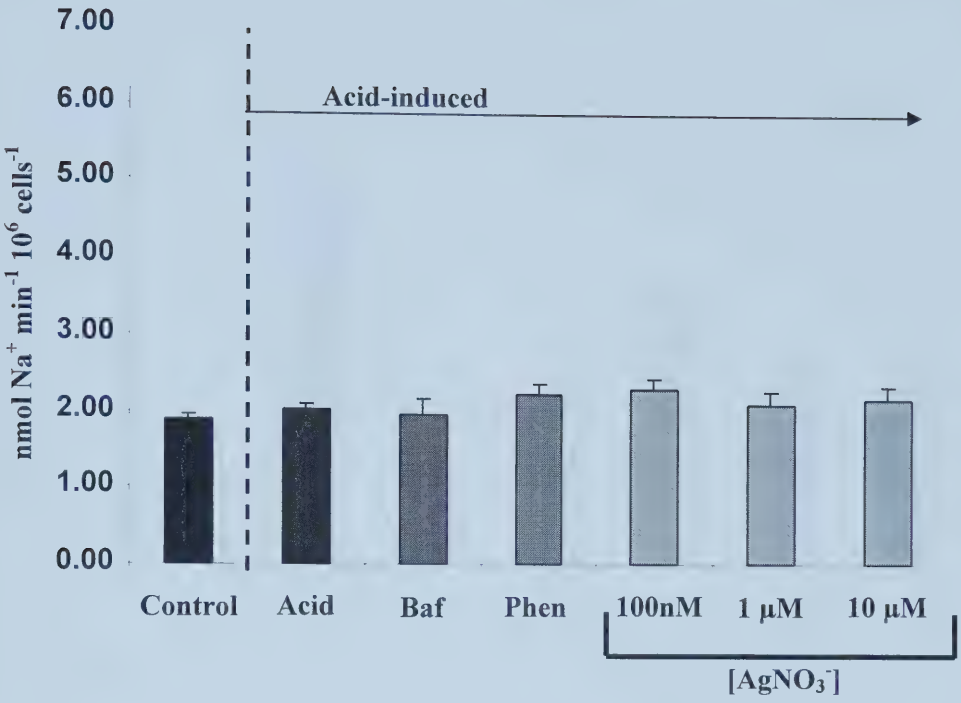

Figure 4.1

Sodium influx rates using $^{22}Na^{+}$ in the pavement cell fraction from the1.03-1.06 g/ml Percoll interface. Pavement cells are not MR cells. Treatments indicated were all acid induced with 20 mM butyric acid. $AgNO_3$ was incubated with cells 1 min prior to the 1 min acid incubation. Abbreviations on graph are: Baf – Bafilomycin (10 nM); Phen – Phenamil (10 µM); concentrations shown are for $AgNO_3$ incubated with cells (100nM, 1µM and 10µM). Data presented as means ± 1 SE. (n=3)

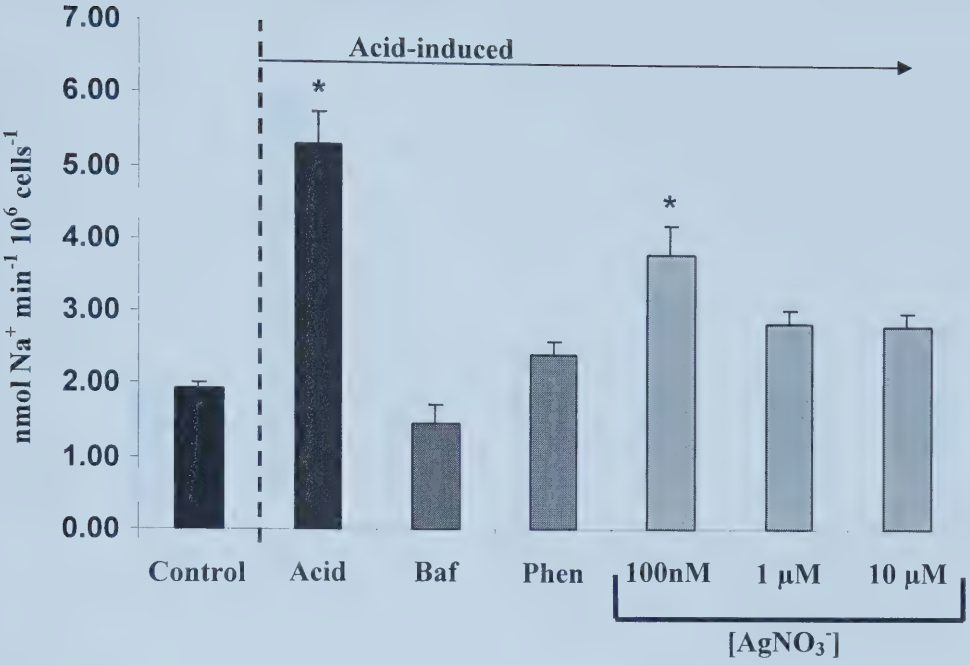

Figure 4.2

$^{22}Na^{+}$ uptake in the total MR gill cell fraction (PNA^{+} and PNA^{-}). All experimental treatments acidified with 20 mM butyric acid. Cells were pre-incubated with appropriate blockers (10 nM Bafilomycin (Baf); 10 µM Phenamil (Phen), or $AgNO_3$ (shown as concentration used on graph)) for one minute prior to $^{22}Na^{+}$ flux measurement. Significance indicated by symbols. Data presented as means ± 1 SE. (n=5-8)

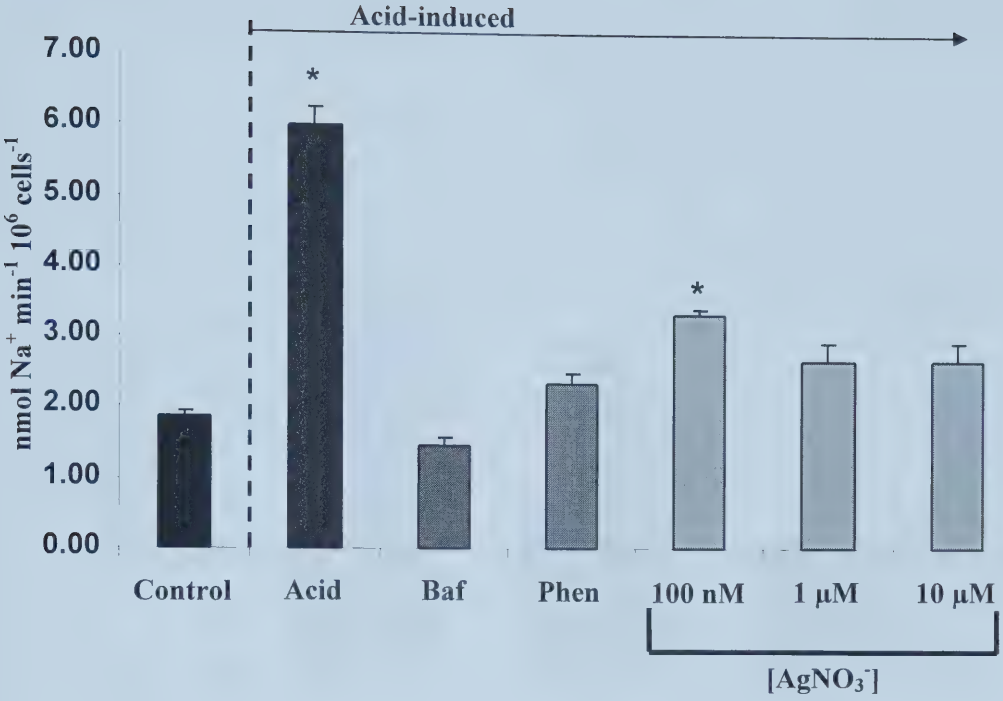

Figure 4.3

$^{22}Na^{+}$ uptake in the PNA^{-} MR gill cell fraction, isolated using total MR cells and the MACS system. All experimental treatments acidified with 20 mM butyric acid. Cells were pre-incubated with appropriate blockers (10 nM Bafilomycin (Baf); 10 µM Phenamil (Phen) and $AgNO_3$ (shown as concentration used on graph)) for one minute prior to $^{22}Na^{+}$ flux measurement. Significance indicated by symbols. Data presented as means ± 1 SE. (n=5-8)

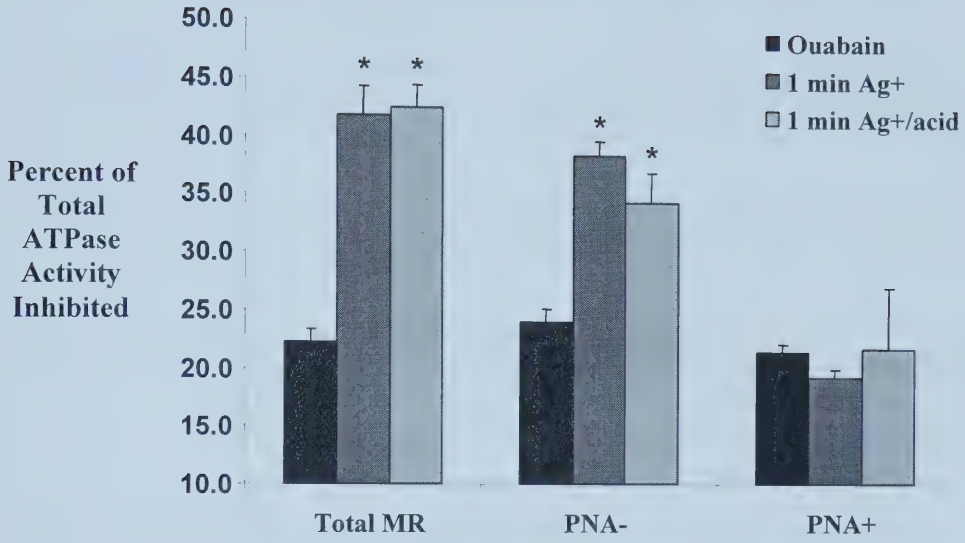

Figure 4.4

The percent of total ATPase activity inhibited by the addition of either ouabain (500 μM), 10 μM $AgNO_3$ alone, or with $AgNO_3$ and acid (20 mM butyric acid) to cause internal acidification, to the total MR cells and the PNA separated fractions. Incubation times with $AgNO_3$ were 1 minute. Significance indicated by symbols. (n=5-6).

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Chapter 5: General Discussion

The original model for ion uptake and acid/base regulation involved a single fish gill MR cell (see Figure 1.1). Since the discovery of Pisam and colleagues (Pisam *et al.,* 1988) of two distinct chloride cells, there has been compelling evidence for the separation of Na^+ uptake/H^+ excretion and Cl^- uptake/HCO_3^- excretion on separate cell types (Reid *et al.,* 2003; Galvez *et al.,* 2002; Goss *et al.,* 2001; Wilson *et al.,* 2000a; Sullivan *et al.,* 1995; Goss *et al.,* 1992). The work included in this thesis has helped to demonstrate at least two morphological and functionally distinct MR sub-populations from the gills of freshwater- and seawater-adapted fish.

Given the evidence accumulated in this thesis, and by other researchers, I have revised the initial model of the fish gill to separate the function of the Na^+ transporting cells and the Cl^- transporting cells. I believe the current model for ion exchange and acid/base regulation in the cells of freshwater fish should link Na^+ uptake to a rheogenically coupled H^+-ATPase on one cell type and Cl^-/HCO_3^- exchange on another (Figure 5.1). In the freshwater environment there is a gradient for Na^+ and Cl^- from the fish into the dilute medium. This requires the fish gill to be able to uptake these salts from the water and transport them into the fish. Acid excretion is linked to Na^+ uptake in the freshwater gill cell model, and our evidence has shown that the PNA^- fraction demonstrates exclusive acid-induced Na^+ influx (Reid *et al.,* 2003), significant loss of H^+-ATPase activity during seawater acclimation (Chapter 3) and increased expression of H^+-ATPase during acidosis (Chapter 2). This is strong evidence that the PNA^- cells are the location of the H^+-ATPase linked to an epithelial Na^+ channel. This would imply that the PNA^+ fraction

is the likely location of the HCO_3^- secreting cells, as evidence does not implicate this cell type in acid secretion or acid-induced Na^+ uptake. However, the lack of a specific pharmacological tool for differentiation of an apical *versus* a basolateral transporter does not allow us to definitively ascribe apical AE function to the PNA^+ MR cells.

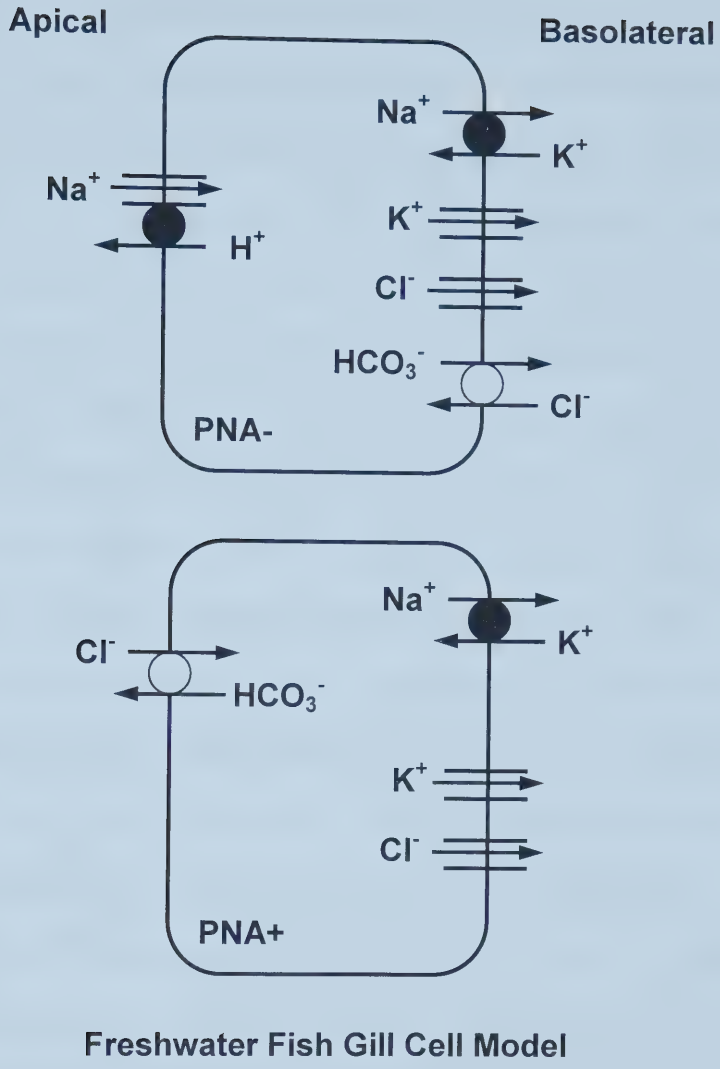

Figure 5.1

Gill cells represented by rectangular structures and showing principal transporters involved in acid/base regulation and the ion uptake associated with this process. Solid circles represent transport requiring ATP and open circles represent exchangers. Channels represented by two lines through membrane.

Unlike the freshwater environment, where the fish has higher concentrations of salts than the medium, seawater contains much higher amounts of salts than the fish, resulting in a gain of ions, and a need to remove these extra ions (salt) from the body. For removal of excess salts from the fish the NKCC co-transporter helps create a gradient for Cl^- to leave the cell on the apical side through a Cl^- channel. The movement of Cl^- helps make the apical side slightly negative and draws Na^+ out between the cells (paracellular) (see review by Marshall, 2002). For acid/base regulation in a saltwater environment, it would not be energetically favorable to primarily use a Na^+ channel linked to an H^+-ATPase to remove excess protons and drive uptake of Na^+, as there is already an existing Na^+ gradient into the fish. Although the precise mechanism is unknown, the removal of H^+, or uptake of Na^+, could be accomplished by using an NHE mechanism that would not require the use of ATP to run. Previous works by Wilson *et al.* (2000b) and Claiborne *et al.* (1999) have suggested that there is an NHE located in the gill cells, although the exact location or isoform still remains unknown (for review see Claiborne *et al.,* 2002). Given this, based on our separation terminology, the model for seawater ion exchange in the gills is slightly different than the freshwater model in that apical H^+ excretion is accomplished *via* an electroneutral NHE type mechanism located on the PNA^- cell type (Figure 5.2).

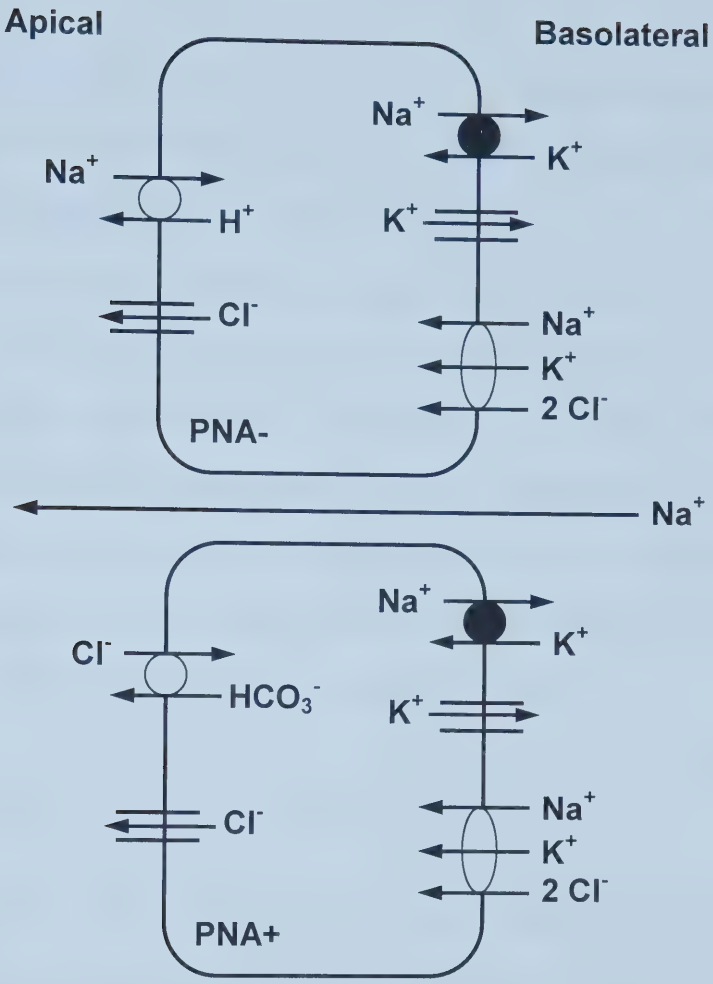

Figure 5.2

Gill cells represented by rectangular structures and showing principal transporters involved in acid/base regulation and the ion uptake associated with this process. Solid circles represent ATPase transport and open circles represent exchangers. Channels represented by two lines through membrane. Arrow between cells shows paracellular movement of Na^+.

The ATPase data from this study (Chapter 3) presented some interesting and predicted results relating to H^+-ATPase. Specifically, we had hypothesized that H^+-ATPase would be reduced in the PNA^- cells with a transition from freshwater to seawater as the proton linked Na^+ channel mechanism would no longer be energetically feasible for the fish in the seawater environment. However, we were surprised at the relatively high levels of H^+-ATPase activity found in the freshwater PNA^+ cells and the concomitant reductions that occurred during seawater acclimation. This current data, along with other research in the field, has led to a further modification of the current model of freshwater acid-base regulation in fish whereby the H^+-ATPase is involved in Cl^- uptake from freshwater.

A revised model for acid-base regulation in freshwater fishes

The appearance of elevated levels of H^+-ATPase in both MR cell sub-types and the concomitant changes found during seawater acclimation suggests that we revisit and revise the current model of acid-base regulation in the fish gill. Figure 5.3 demonstrates our model based on current evidence from both freshwater and seawater adapted fish and other model acid-base regulating tight epithelia (e.g. turtle bladder and mammalian inner medullary collecting duct of the kidney).

In freshwater, the PNA^- cell type is responsible for Na^+ uptake as demonstrated in the paper by Reid *et al.* (2003). Acid-stimulated, phenamil-sensitive Na^+ uptake could only be found in the PNA^- cell fraction and not in the PNA^+ fraction. Theoretically, Na^+ uptake from low ionic strength media requires active H^+

extrusion to the water via an apical H^+-ATPase to provide the driving gradient for Na^+ uptake. This mechanism for Na^+ uptake was first proposed by Avella and Bornancin (1989) based on the frog skin model of Na^+ uptake (Harvey and Ehrenfeld, 1988; Harvey and Ehrenfeld, 1986; Ehrenfeld *et al.,* 1985). A similar mechanism has also been demonstrated for acid-base regulation in the mammalian kidney (Brown and Breton, 2000). In the kidney, two types of MR cells exist, termed α- and β- (acid and base secreting) IC cells. These cells have similar morphological and physiological characteristics to the MR cells in the fish gill. The results of this and previous experiments are consistent with this model and allow us to term the PNA^- cell the α-MR cell in the fish.

During seawater acclimation, external Na^+ rises to a level that favours inward directed Na^+ movement without the need for an energetically expensive apical H^+-ATPase. Our results, showing a reduction in PNA^- (α-MR cell) H^+-ATPase during seawater acclimation are consistent with this model. Other authors (Piermarini and Evans, 2001; Lin *et al.,* 1994) have also documented reduction in H^+-ATPase immunoreactivity during seawater acclimation and also the appearance of immunoreactivity of the NHE-2 isoform of the Na^+/H^+ exchanger family in seawater adapted fish (Wilson *et al.,* 2002).

Our recent findings of elevated H^+-ATPase activity in PNA^+ cells was unexpected, as were the changes that occurred during seawater acclimation. Re-evaluation of the current model in light of these and other results in the literature leads us to suggest that H^+-ATPase plays a significant role in Cl^- uptake and acid-base regulation in freshwater fish. Cl^- uptake from very dilute freshwater does not have a

favourable driving electrochemical gradient from the water to the fish. Similarly, a strict gradient for HCO_3^- from the blood to the water is not sufficient to provide a driving gradient for HCO_3^- excretion under normal conditions. We propose that Cl^- uptake is driven by a functional coupling of a basolateral H^+-ATPase, an apical Cl^-/HCO_3^- exchanger (molecular identity as yet undetermined) and carbonic anhydrase (CA). This metabolic unit, functions by actively secreting H^+, derived from the hydration of CO_2 by the enzyme CA, through the basolateral H^+-ATPase. CA has been demonstrated to be expressed in high levels in the MR cells of freshwater fishes (Gilmour *et al.,* 2002; Sender *et al.,* 1999; Flugel *et al.,* 1991; Rahim *et al.,* 1988) and this is expected, as CA is necessary for cells involved in acid-base transfer by supplying the counterions for transport. Unfortunately, the heterologous anti-human carbonic anhydrase II (CAII) antibodies that we were able to obtain did not cross-react with our isolated fractions so we could not demonstrate this in our isolated MR cells populations.

CA has been demonstrated to be physically associated with the c-terminus of many isoforms of the HCO_3^- transporting superfamily (Vince and Reithmeier, 2000; Fierke *et al.,* 1991; Srere, 1985). The action of CAII in the presence of CO_2 would create a local, short lived elevation in HCO_3^- in the region of the pore of the anion exchanger, raising $[HCO_3^-]$ values in excess of 4 mol/L (Goss, 2003). A closely associated H^+-ATPase on the basolateral membrane is necessary to remove the H^+ generated from the CO_2 hydration reaction and remaining after HCO_3^- transfer across the apical membrane by the AE. This elevated $[HCO_3^-]$ will provide a sufficient gradient for net electroneutral outward HCO_3^- movement and drive anion uptake from

dilute freshwater. This model is supported by a variety of experimental evidence from other researchers but has not been presented in this manner before. Early on, the laboratories of Jean Maetz, Michelle Bornancin and Frank Epstein suggested that there was a specific Cl^-/HCO_3^- ATPase activity in membrane vesicles from freshwater fish (Naon *et al.,* 1981; Bornancin *et al.,* 1980; DeRenzis and Bornancin, 1977; Maetz, 1976; Epstein *et al.,* 1973), a finding which could also be explained by our revised freshwater gill cell model where the H^+-ATPase is used to functionally drive Cl^- transport. More recently, Wilson *et al.* (2000b) have demonstrated both H^+-ATPase and CAII staining in the apical MR cells of freshwater mudskippers, but did not perform immunogold analysis to examine the precise localization. The basolateral membrane in MR cells is known to have extensive infoldings that can place Na^+/K^+-ATPase at the sub-apical area (Wilson *et al.*, 2000b; Philpott, 1966). Our revised freshwater gill cell model allows one to postulate the reasoning for these extensive basolateral infoldings is the necessity for very close proximity of the basolateral membrane to the apical membrane. Structure-function relationships form the basis for understanding physiological function. We believe that in PNA^+ β-type MR cells, the H^+-ATPase can be located sub-apically (but on the basolateral membrane) in close approximation to the apical AE/CA complex forming the complete Cl^- uptake mechanism. Evidence to support this hypothesis has recently been published by Katoh and Kaneko (Katoh *et al.,* 2003) who, using immunogold analysis, show sub-apical, but no apical, staining of the H^+-ATPase in MR "chloride cells" of killifish. During seawater acclimation, external $[Cl^-]$ (~450 mM) rises to approximately 5 - 7 times the internal $[Cl^-]$ based on an estimated 60 - 80 mM Cl^-

inside MR cells. This elevation would reduce but not eliminate the thermodynamic requirement for Cl^- uptake for acid-base regulation, as the counter-ion (HCO_3^-) is also elevated in seawater and a predicted maintained membrane potential of approximately -60 mV will counter passive Cl^- movement. It is important to recognize that although the primary function of MR cells in seawater is Cl^- secretion, a continued small Cl^- uptake in exchange for HCO_3^- is necessary for acid-base regulation. The reduced energy requirement resulting from a reduced gradient for Cl^- uptake would be predicted to decrease, but not eliminate H^+-ATPase activity, as H^+-ATPase activity is also required by many intracellular organelles for proper functioning. The predicted result is a reduction in H^+-ATPase activity in PNA^+ β-MR cells during seawater adaptation, in agreement with the results generated in this study (Chapter 3).

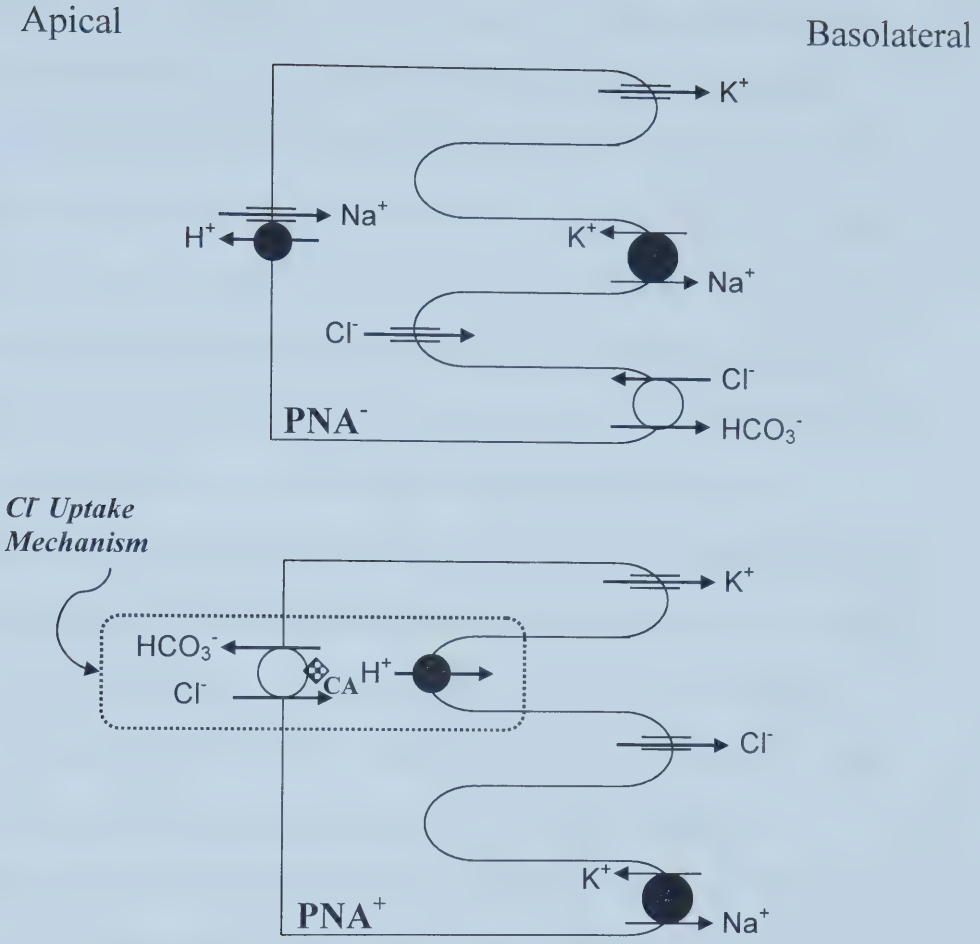

Figure 5.3

Updated freshwater fish gill cell model. PNA^- and PNA^+ cells marked on figure. Open circles represent exchange, filled circles represent active transport and channels represented by double lines through membranes. Hypothesized Cl^- uptake mechanism marked by dashed line on PNA^+ cell type. Small checkered square attached to AE on PNA+ cell represents carbonic anhydrase (CA). Apical and basolateral surfaces indicated at top of graph.

Despite a large amount of evidence to support our revised model of the fish gill, many aspects remain to be specifically identified and supported. The identity and function of the apical AE is as yet unknown. Recently, the Slc26a6 AE Pendrin has been demonstrated to be the apical AE in the β-IC cells of the mammalian kidney (Royaux *et al.*, 2001; Soleimani *et al.*, 2001). Immunocytochemical localization of Pendrin-like immunoreactivity has been demonstrated for euryhaline stingrays (Piermarini *et al.*, 2002), but has not yet been performed for salmonids. Furthermore, association of CA with the apical AE remains to be demonstrated. However, evidence for this association in other AE is rapidly accumulating (Vince *et al.*, 2000; Vince and Reithmeier, 2000; Vince and Reithmeier, 1998) and we have no reason be believe that this association will not hold true for other members of the HCO_3^- transporting superfamily. Our future research focus will take advantage of our ability to isolated MR cells into separate populations and to use this technique to functionally characterize these individual cell populations. The power of this approach is demonstrated in the above results, whereby independent changes in biochemical activity in separate cell types are unmasked by the ability to separate the cells.

Metals toxicology

One problem with the hypothesis of uptake of silver affecting Na^+/K^+-ATPase is that the silver is taken up from the water by the apical membrane and must traverse the cytoplasm to reach the basolaterally located Na^+/K^+-ATPase to exert its toxic

action. Because of the reactivity of the positive silver ion to Cl^- to form AgCl precipitates, and the overall negative charge inside the cell, the half life of free Ag^+ in this high Cl^- solution inside the cells would be extremely small. However, based on the ultrastructure of the cell, it has been suggested that there could exist tubular formations (part of endoplasmic reticulum) that would act like a transcellular "freeway" from one side of the cell to the other, bypassing the majority of the cytoplasm, allowing free Ag^+ to affect Na^+/K^+-ATPase and other ATPase functions on the basolateral membrane of the cell (Mollgard and Rostgaard, 1978). Also, compartmentalized transport has been proposed in other cell types. For example, in fused renal epithelia cells the uptake of Na^+ has been suggested to occur into the nucleus of the cell before distribution out to the cytoplasm. This mechanism has been explained by having heterogeneous distribution of transport enzymes working in conjunction with specific three dimensional cell conformations (Oberleithner *et al.*, 1992). These theories help explain why previous research has demonstrated that Ag^+ seems to exert its affect on the Na^+/K^+-ATPase (Morgan *et al.*, 1997), but does not help explain why we get a larger inhibition of total ATPase activity with incubation of Ag^+, than is seen with the addition of ouabain alone. One possible explanation is that we digest and separate the MR cells in our protocol. The separation of these cells causes them to lose polarity, and most likely the cell conformation they have when situated in an epithelial layer. We can see this affect when the cells are viewed under microscopy. The separated gill cells are round and swelled, not like the typical view when looking at an epithelial sheet. The crypts and infoldings are not seen on separated cells. Our protocol most likely disrupts the geometric conditions of cells *in*

vivo. If this is the case, then we are not seeing compartmentalized transport in these cells, although it may exist *in vivo*. This would explain why we are seeing a much greater inhibition of total ATPase activity in our separated cells with the addition of Ag^+, as the Ag^+ can now access the entire cytoplasm and bind non-specifically to negative charges found anywhere within the cell. The Ag^+ may not be binding to just Na^+/K^+-ATPase, but other proteins with exposed negative sulfhydryl groups, causing them to malfunction as well. This data may be inconsistent with previous research showing the affects of Ag^+ toxicity on Na^+/K^+-ATPase, but shows that Ag^+ has the ability to affect more than one enzyme and its toxic affect might not be specific, or totally attributable, to Na^+/K^+-ATPase within the cell.

Future Directions

The novelty of this research has been the ability to isolate functionally distinct sub-types of MR gill cells. This technique has many implications for future studies involving MR cell ion and acid/base regulation. Some future directions could include:

1. Employing the PNA separation technique to isolate PNA fractions to extract mRNA cloning of transporters, such as the apical AE from the PNA^+ cells. This research has lagged, most likely from the inability to previously isolate pure populations of gill MR cell sub-types.
2. Anion channels in seawater acclimated fish are postulated to exist (CFTR), but no evidence as of yet has been shown. Our isolation technique could yield a pure population of PNA^+ cells (believed to be the base secreting cells) that could allow for patch clamping analysis.
3. Analysis of whole gene expression using microarray analysis of the PNA^- and PNA^+ populations and changes during environmental stresses and toxicological assessment.

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